MIXED INFECTIONS OF MAIZE DWARF MOSAIC VIRUS AND CUCUMBER MOSAIC VIRUS IN MAIZE

BY

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NOTE: The maize cultivars used in this work have been coded. In the text they are referred to by these codes as their identity was not important in this investigation. A maize type suitable for use in the programme was necessary and an unbiased screening of maize types was carried out. The maize cultivars/hybrids used in this programme, when coded, are identified in IX.C.

ABBREVIATIONS

AA	Amino acid
Ab	Antibody
As	Antiserum
BSA	Bovine serum albumin
BMV	Brome mosaic virus
CMV-K	Cucumber mosaic virus strain K
CMV-Is	Cucumber mosaic virus strain Israel
CMV-Tob	Cucumber mosaic virus strain Tobacco
cv.	Cultivar
da	Daltons
DAPI	Diamino phenyl indole
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
DEAE	Diethylaminoethylcellulose
DIECA	Diethyldithiocarbamate
EDTA	Ethylene diamine tetra-acetic acid
FA	Formaldehyde
FDA	Fluorescein diacetate
FITC	Fluorescein isothiocyanate
F/P ratio	Fluorescein/protein ratio
GAR	Goat anti-rabbit serum
HEPES	N-2 hydroxylethyl piperazine N'2 ethane sulphonic acid
H.S.	High speed centrifugation
IEB	Immunoelectroblotting test
IEM	Immunosorbent electron microscopy
IgG	Gamma-globulin fraction of serum
Kv	Kilovolts
L.S.	Low speed
MDMV	Maize dwarf mosaic virus
M_r	Molecular weight
OD_{260nm}	Optical density (absorbance reading at indicated wavelength)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PC	Phosphatidylcholine
PS	Phosphatidylserine
PC:chol	Phosphatidylcholine:cholesterol
PS:chol	Phosphotidylserine:cholesterol
RhPV	Rhopalosiphum padi virus
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
ss-RNA	Single stranded ribonucleic acid
Temed	N N N' N' tetramethylethylenediamine
TMV	Tobacco mosaic virus
Tris	Trishydroxymethylaminomethane
w/v	weight/volume ratio
v/v	volume/volume ratio
S_{20w}	Sedimentation coefficient in Svedbergs
UV	Ultraviolet

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SUMMARY

Maize plants collected in three geographically distinct regions of South Africa were found to be doubly infected with maize dwarf mosaic (MDMV) and cucumber mosaic virus (CMV). A mixed infection of these two viruses could be maintained in maize plants grown under laboratory conditions. The possibility of synergism or of an interference mechanism between MDMV and CMV in dual infections was investigated and it was found that prior infection with CMV interfered with subsequent infection by MDMV. MDMV and CMV were shown to be non-persistently transmitted by Myzus persicae, Rhopalosiphum padi and Rhopalosiphum maidis aphids. Protoplasts were isolated from maize seedlings and could be viably maintained for up to 66 hours. The maize protoplasts were infected with CMV and MDMV either singly, or together as a mixed inoculum. Infection curves for each virus were plotted. The presence of CMV in a mixed inoculum appeared to prevent infection of the protoplasts by MDMV. Protoplasts were isolated from plants systemically infected with CMV and/or MDMV. Superinfection of protoplasts prepared from CMV infected seedlings with MDMV was not possible. As a possible vehicle for virus infection of protoplasts liposomes were produced. Initially fluorescent dyes were incorporated in them. These were fused to the maize protoplasts. Attempts were made to encapsulate virus particles in the liposomes and fuse them to maize protoplasts but this was not successful.

CHAPTER I

INTRODUCTION

In January 1984, maize plants with unusual symptoms were noticed during a field trip in maize growing areas in South Africa. The symptoms, interrupted streak and mosaic, were not characteristic of single virus infections previously known to occur in maize in South Africa (von Wechmar, pers. comm.). Three samples, one from Natal and two from the Transvaal, were targeted for further investigation. A mixed infection of two or more viruses was suspected. Biophysical and serological techniques were employed to identify the two viruses involved. Preliminary findings implicated maize dwarf mosaic virus (MDMV) and cucumber mosaic virus (CMV).

MDMV and CMV infections of South African crops had previously been studied in this Department (Chauhan, 1985 and Lupuwana, 1985). In addition CMV had been found to be seedborne in two maize cultivars (Knox, 1983). A series of biological tests were undertaken in an attempt to answer questions such as whether the mixed infection of these two viruses could be maintained under laboratory conditions, and what the epidemiological implications of double infections could be. This is reported in Chapters III, IV and V of this thesis.

The problem of mixed infection in the field was viewed from a different angle by studying a possible interaction of the viruses at a cellular level in maize protoplasts. Protoplasts have often been used to study various aspects of viral replicaton since they are believed to simulate the situation, which occurs in whole plants, at the level of a single cell. In addition, viral replication is essentially synchronous in protoplasts and their easy disruption permits separation of viral proteins and nucleic acids and their analysis. Barley protoplasts had previously been produced in the Department. Conditions for the isolation of maize protoplasts had to be optimized. Factors such as the maize cultivar from which protoplasts could be prepared and incubation conditions had to be carefully considered before the infection of the protoplasts with MDMV and CMV could be attempted. Once these variables had been optimized, infection methods and subsequent techniques for detection of the viruses within the protoplasts could be applied. This work is reported in Chapter VI.

Liposomes have frequently been used for infection of plant protoplasts with viruses and their RNA. The use of liposomes is known to enhance the efficiency of infection. The possibility that liposomes could be used in the infection of maize protoplasts was therefore investigated (Chapter VII).

CHAPTER II

LITERATURE REVIEW

A. CUCUMBER MOSAIC VIRUS

According to Francki and Hatta (1980), the occurrence of cucumber mosaic virus (CMV) was first reported by Doolittle and Jagger in 1916. In recent years it has been described as one of the most 'cosmopolitan' viruses known (Francki and Hatta, 1980).

1. Introduction

Cucumber mosaic virus is a member of the cucumovirus group which also includes tomato aspermy virus (TAV) and peanut stunt virus (PSV). It has recently been suggested that the Cucumoviruses, the Bromoviruses and Ilarvirues be collectively grouped into the family "Tricornaviridae" since they share several properties (van Vloten-Doting et al., 1981). However, this proposal has not been favoured by the ICTV (Francki, 1985).

Table II.1 presents some of the major physical and biochemical properties of CMV. Kaper et al. (1965) were the first to discover that CMV had a divided genome. Peden and Symons (1973) and Lot et al. (1974) showed that the

multipartite genome was packaged in isocapsidic particles. These workers found that three virus particles were necessary for infection; two particles containing RNA 1 and 2, and a third particle containing RNA 3 and subgenomic RNA 4. A fifth RNA species, a satellite-like RNA known as CARNA-5 may also be found in some CMV strains (Kaper and Waterworth, 1981).

The possible advantages and disadvantages of having a multipartite genome are discussed by Fulton (1980). For infection to occur, the complementary particle types of multicomponent viruses such as CMV must infect a cell simultaneously. This may affect the efficiency with which CMV infects host cells. This does not appear to be a problem in natural transmission of CMV by aphids, since a large number of aphid species transmit the virus successfully. Another advantage of a multipartite genome is that a source of variation could be provided since reassortment of characteristics could easily occur. This has been suggested as a reason for the existence of divided genomes (Fulton, 1980). It has also been proposed that, as the genome size is relatively large, for stability of the nucleic acid it is advantageous for the genome to be divided into different segments. The small particle size could also possibly facilitate cell to cell spread of whole capsids through the plasmodesmata (Fulton, 1980).

Table II.1: Physical and Biochemical Properties of cucumber mosaic virus.

			REFERENCE
A.	PARTICLES		
	Shape	Isometric	Finch et al., 1967
	Size	28-30 nm	"
	Morphology	icosahedral	"
	Sedimentation coefficient in S_{20w}	98,6 (-1,04c) (c= virus concentration (mg/ml))	Francki et al., 1966 van Regenmortel, 1967 Francki et al., 1979
	UV absorption coefficient $E^{0,1\%}_{260\ nm}$	5	Francki et al., 1979
B.	VIRAL PROTEIN		
	Molecular weight	2,4 - 2,6 x 10^6 da	Van Regenmortel, 1972 Lupuwana, 1985 Symons, 1985
	Amino acids	226	Mossop et al., 1976
	Polypeptides per particle	180	Finch et al., 1967 Habili and Francki, 1974
C.	NUCLEIC ACID		
	Type	linear, positive sense RNA	Kaper and West, 1972
	Molecular weight	RNA 1 - 1,35 x 10^6 da RNA 2 - 1,16 x 10^6 da RNA 3 - 0,85 x 10^6 da RNA 4 - 0,35 x 10^6 da (RNA 5 - 1 x 10^5 da)	Kaper and West, 1972 Peden and Symons, 1973 Lot et al., 1974 Mossop and Francki, 1977 Gould et al., 1978

2. Host Range and Symptoms

The virus is probably best known for its broad host range. Both dicotyledonous and monocotyledonous plants have been reported to be susceptible. In all some 775 plant species from 85 plant families are known to be hosts for CMV (Lovisolo, 1980). CMV is known to infect many agricultural and horticultural crops (Waterworth, 1981; Alberts *et al*, 1985; Lupuwana, 1985). It is commonly found in the families Cucubitaceae and Solanaceae. Kaper and Waterworth (1981) give an extensive list of crops which are susceptible to CMV. Von Wechmar *et al*. (1984) found CMV associated with smallgrains in South Africa. Lupuwana (1985) showed that CMV was seedborne in *Lupinus angustifolius*. In a recent survey of crops in the Transvaal Lowveld, von Wechmar (pers. comm.) identified CMV in diseased cucumbers, squash, green pepper, grenadilla, tomato and some common weeds.

Although CMV has a wide host range, it has not normally been associated with maize disease. Holdeman and McCartney (1965), in a review of virus diseases in corn, report that the virus produced numerous, light-coloured, yellow elliptical spots of various lengths and widths, forming stripes parallel to veins and occasionally a mosaic type mottle, similar to sugarcane mosaic. In addition, striping and a tendency for the leaves to split was noted. Dwarfing and even death of seedlings may occur when infected with CMV.

According to Holdeman and McCartney (1965) CMV in maize has been associated with local epidemics in vegatable crops. Tien Po et al. (1982) first isolated CMV-K from cornflowers in China and mention that this isolate is readily distinguishable from other CMV isolates by its ability to infect maize. In a laboratory study, Rao and Francki (1982) infected Zea mays with three strains of CMV; CMV-U, CMV-M and CMV-K. They found that only CMV-K infected maize, apparently causing a mosaic symptom.

Of agricultural importance is the fact that CMV can be harboured in several weed species (Tomlinson and Carter, 1970). The following are some common weeds occurring naturally in maize crops in South Africa: Datura stramonium, Amaranthus spp., Commelina benghalensis, Xanthium strumarium, Physalis angulata, Senecio vulgaris, Chenopodium spp. and Portulacca oleracea (Grabant, 1985). Von Wechmar (pers. comm.) showed that C. benghalensis collected from two different maize fields was infected with CMV. Whether the other weeds act as alternate/reservoir hosts to CMV in South Africa has not yet been determined.

CMV often produces no apparent symptoms in the host it infects. Thus this source of virus may often be overlooked in the agricultural situation (Tomlinson and Carter, 1970; Bruckart and Lorbeer, 1976).

Symptoms caused by CMV are ubiquitous and range from no obvious symptoms to plant death (Smith, 1972). Some CMV strains produce reactions not readily distinguishable from those caused by totally unrelated viruses (Francki and Hatta, 1980). It is obvious that CMV was first noticed in cucurbit plants and derives its name from the characteristic symptoms which it produces in cucumber.

Rao and Francki (1982) investigated genetic aspects of symptom expression by constructing eighteen pseudorecombinants from three strains of CMV and infected ten host plant species with them. They found that symptom expression is controlled by either a single RNA segment or an interaction between two or more virus genome segments. They further noted that it involves both the host plant's genetic material as well as that of the infecting virus. The presence of CARNA 5, a small single-stranded RNA molecule of 1×10^5 daltons, has been found to be an additional factor involved in symptom variation. The influence of CARNA-5 on symptom expression is reviewed by Kaper and Waterworth (1977).

3. Transmission of CMV

CMV is aphid-, seed-, sap- and dodder-transmissible (Bos and Maat, 1974; Bouwen et al., 1978; Hamilton, 1985).

a) Aphid transmission

At least sixty aphid species have been reported to transmit CMV in a non-persistent manner. The most common aphid species involved in CMV transmission were found to be Myzus persicae and Aphis gossypii (Francki et al, 1979).

Normand and Pirone (1968) found that there was differential transmission of some strains of CMV when they occurred together in a plant. They showed that when four strains of CMV were present in a tobacco plant, two strains were readily transmitted from tobacco to tobacco, but the two other strains were rarely transmitted. Gera et al. (1979) showed that the composition of the coat protein determined which strains of CMV were aphid transmissible. An interaction between the coat protein and the aphid stylet or foregut is thought to occur (Hamilton, 1985). The type of host plant on which the aphid species has been propagated, the concentration of virus in the host plant and the presence of a helper or inhibitory factor all contribute to the rate of aphid transmission (Bouwen et al., 1978; Francki et al., 1979; Gera et al., 1979; Hamilton, 1985). RNA 3 may also play a role in aphid transmissibility of CMV (Mossop and Francki, 1977).

b) Seed transmission

CMV may be found in the seeds of certain crop plants such as Cucurbita spp. (squash), Phaseolus vulgaris (common bean), Cucumis spp. (cucumber) and Lupinus luteus (Hamilton, 1985). CMV has also been reported in the seed of barley (von Wechmar et al., 1984) and maize (Knox and von Wechmar, 1984). In addition, Tomlinson et al (1970) showed that CMV may be seedborne in Stellaria media, and Quiot (1980) found that the virus may persist in the seed up to 21 months after the weeds have been removed. Germination of the seeds provides infection foci for further spread of CMV by aphids. An indication of the seriousness of this finding is demonstrated if some calculations are considered. If only 1% of the S. media seeds are infected and of these there is a 10% emergence, there will be one infected seedling per square yard of field. Thus, small aphid infestations may cause serious outbreaks with the presence of so many virus infection foci.

In essence, a natural reservoir for virus perpetuation and spread is provided by seedborne virus (Tomlinson, 1975; Quiot, 1980; Martelli and Russo, 1985).

4. Serology

The serology of CMV has been extensively studied. Scott (1968) found that some strains of CMV are poor immunogens. Devergne and Cardin (1973; 1975) serologically classified strains of CMV using gel double diffusion tests. On the basis of spur formation in the agar, they grouped the CMV isolates into four serological types. It should be noted however that serological studies of CMV depend greatly on the physical state of the virus, the diffusion medium and the ratio of antigen to antibody (Kaper and Waterworth, 1981).

The extreme instability of CMV causes complications in the study of serological relationships (Devergne and Cardin, 1973). Francki and Habili (1972) found that the instability of CMV could be overcome by treating the immunogen with 2% formaldehyde (FA) or gluteraldehyde (GA). They showed that this treatment causes cross-linkage between the protein and RNA molecules, decreasing the possibility of the virus dissociating into its component parts. Van Regenmortel (1972) found that FA treatment did not alter the antigenicity of the virus.

B. MAIZE DWARF MOSAIC VIRUS

Maize dwarf mosaic virus (MDMV) infections in maize in South Africa were investigated in detail by Chauhan (1985). Only a brief outline of some of the more important characteristics of this virus will be given.

MDMV is a filamentous virus and a member of the Potyvirus group. Sugarcane mosaic virus (SCMV) and MDMV are generally believed to be strains of the same virus (Pirone, 1972) and they have worldwide distribution (Gordon et al., 1981). The serological relationship between the two viruses has been a source of controversy for several years (Shepherd, 1965; Ford and Hill, 1976; Louie and Knoke, 1975). Six strains of MDMV, referred to as MDMV-A to -F, have been reported by Louie and Knoke (1975). Identification of the many strains of MDMV is contentious. Various methods have been used to differentiate them and, in many cases, they have never been compared by one particular group of workers in one laboratory. It is uncertain therefore whether these strains are indeed different, or whether they are merely isolates recorded by different workers. Gillaspie et al. (1984) have reviewed the situation. They have suggested that more uniform criteria for strain identification should be employed and back testing with known strains done to clearly characterize strains/isolates.

Table II.2: Physical and biochemical properties of maize dwarf mosaic virus

			REFERENCE
A.	PARTICLES		
	Morphology	flexuous rods	Bancroft et al., 1966
	Size	11-13 nm diameter 750-755 nm length	Shepherd, 1965
	Sedimentation coefficient	148-170 S_{20w}	Shepherd, 1965; Bancroft et al., 1966; Seghal, 1968; Jones and Tolin, 1972; Langenberg, 1973; Tosic and Ford, 1974
	UV absorption coefficient $E^{0,1\%}_{260}$	2,7	Langenberg, 1973
	260:280 nm Absorbance ratio	1,17-1,26	Seghal and Jean, 1968 Langenberg, 1973
B.	VIRAL PROTEIN		
	Molecular weight	$36,5 \times 10^3$ (SDS-PAGE)	Hill et al., 1973
		$28,5 \times 10^3$ (AA-analysis)	"
	Amino acids	264	Hill et al., 1973
		290	Hollings and Brunt, 1981
C.	NUCLEIC ACID		
	Type	Single stranded positive sense RNA	Hollings and Brunt, 1981
	% RNA	6%	Hill et al., 1973
	Molecular weight	$2,7 \times 10^6$ da	Pring and Langenberg, 1972

MDMV has been shown to infect maize, sugarcane and sorghum as well as other members of the Gramineae family (Pirone, 1972; Chauhan, 1985). Some 300 grass species have been reported to be susceptible to infection by MDMV (Rosenkranz, 1983). MDMV infection of maize usually induces the appearance of mosaic symptoms and dwarfing of the plant may occur (Williams and Alexander, 1965; Seghal and Jean, 1968; Tosic and Ford, 1972; von Wechmar and Chauhan, unpublished).

MDMV is transmitted in a non-persistent manner by at least 20 different aphid species (Knoke and Louie, 1981). Bancroft et al. (1966) showed that the efficiency of MDMV transmission varies between aphid species. These workers reported that Dactynotus spp. was the most efficient vector of MDMV. Aphid transmission of the virus from seedlings, originating from infected seeds, can lead to its secondary spread. In 1982 Straub reported that when young maize seedlings were infected and aphids were present, 100% infection and an 80% yield reduction of the maize crop resulted. Yield losses, due to MDMV infection alone, of 10-54% have been reported (Gordon et al, 1980). In South Africa yield losses due to MDMV infections in maize have not been determined.

MDMV has been shown to be seed transmissible. Shepherd and Holdeman (1965) reported MDMV transmission in 0,4% of maize seed tested. Other workers have reported 0,008% and 0,2% seed transmission (Hill et al., 1974; Tosic and Sutic, 1977). Von Wechmar and Chauhan (1984) and Chauhan (1985) showed that mosaic symptoms caused by seedborne virus arose in 1,15% of the 800 seedlings tested. The rate of germination of the seed was related to the presence of virus.

C. MIXED INFECTION OF PLANTS

Mixed viral infections often occur in nature (Kassanis, 1963) and various virus interactions have been reported between such viruses. The type of interaction depends on the type of virus, their relatedness, the relative concentration of each virus and the environment of the doubly infected plant (Gibbs and Harrison, 1976). Examples will be discussed in some detail:

1. Mixed infection of plants with unrelated viruses:

According to Gibbs and Harrison (1976), McWhorter and Price in 1949 showed microscopically that two unrelated viruses could infect and multiply in a single plant cell simultaneously. Since then there have been several reports of mixed infections of plants by unrelated viruses. Symptoms produced in doubly infected plants are often more severe than infection by either virus alone (Kassanis, 1963). Rochow and Ross (1965) showed that when plants were doubly infected with potato virus X (PVX) and potato virus Y (PVY), there was an increased concentration of PVX in the plant than when PVX infected the plant alone.

Calvert and Ghabrial (1983) found that soybean mosaic virus (SMV) and bean pod mosaic virus (BPMV) interact synergistically in soybean plants. There was a higher concentration of BPMV in doubly infected plants than in singly infected plants. In contrast, SMV concentration was similar in doubly and singly infected plants.

Several plant diseases have been shown to be caused by mixed infections. Uyemoto et al. (1981) found that two viruses, maize chlorotic mottle (MCMV) and maize dwarf mosaic virus (MDMV) were the causal agents for corn lethal necrosis disease in maize. Early infections with the two viruses were shown to cause yield losses of 50-90%. Tomato scorch disease in tomatoes was found to be caused by co-infection of plants with tobacco mosaic and potato Y viruses (Clark et al., 1980). The disease spread was found to be associated with prevailing drought conditions which caused aphids to migrate from the dry surrounding vegetation to the lush tomato crop. Natural infection of a crucifer (Brassica oleracea) by turnip mosaic and cauliflower mosaic viruses was reported by Khan (1982) in the southeastern United States where it is an important cash crop. Other virus diseases previously thought to be due to single infection have been found to be caused by more than one virus (Tomlinson and Ward, 1981).

Carr and Kim (1983) compared the ultrastructural response of bean cells to single and mixed infection with cowpea moaic virus (CPMV) and bean yellow mosaic virus (BYMV). An orderly arrangement of aggregates and intranuclear inclusions of virions were observed in doubly infected cells. These were not present in singly infected cells.

2. Mixed infection of plants with related viruses:

When a plant is infected by different strains of one virus, different interactions between the two viruses may occur. Gibbs and Harrison (1976) report that Sadasivan (1940) showed that if a non-lesion strain of TMV is inoculated to a plant together with a lesion-forming strain, there is a decrease in the number of lesions produced. In 1951 Bennett showed that when plants are systemically infected with one strain of virus, they are often protected against super-infection with a second strain.. This phenomenon is referred to as cross-protection and has been used to investigate relationships between different virus isolates. Systemic symptoms in plants infected with two strains of a virus are often intermediate in severity between those caused by infection with each strain alone (Gibbs and Harrison, 1976).

Several suggestions have been made in attempts to explain the mechanism of cross-protection:

a) An exclusion mechanism prevents two strains multiplying in one cell (Rappaport and Wu, 1962). When two viruses reach an infection site it was thought that neither could initiate infection unless the one or other virus was inactivated.

b) The occurrence of particles with mixed coat proteins in doubly infected plants has led to the idea that the RNA of the second virus becomes coated with free coat protein of the first inoculated strain and is thus sequestered (De Zoeten and Fulton, 1975).

c) Inactivation of RNA of the challenging strain by virus specific RNA replicase binding to it could occur. This would not happen with unrelated viruses which had different replicases (Gibbs, 1969).

d) An inhibitory substance is produced by one strain so that there is no replication of the challenging strain (Matthews, 1981).

e) Barker and Harrison (1978) proposed that there is competition by the two viruses for materials and sites in the cell. This mechanism would be mediated by RNA polymerase. The RNA of the challenging strain would be able to use the polymerase of the protecting strain, but the former would be at a quantitative disadvantage. This would mean that less RNA and proteins of the challenging strain could be synthesized compared with the protecting strain.

f) Ziemiecki and Wood (1976) proposed that a polypeptide is produced by the protecting strain which alters the specificity of ribosome binding in

favour of viral RNA of the established strain. These workers used radioactive double labelling of protein produced in cucumber cotyledons which were doubly infected with two strains of CMV differing in symptom type. No differences in the protein profiles, which could explain the difference in the symptom expression of each strain, could be detected.

Further work is required before a complete understanding of the phenomenon of cross-protection is achieved.

It is of significance that vector specificity may be altered as a result of mixed infections. Rochow and Gill (1978) showed that transmission by Rhopalosiphum padi aphids of the MAV strain of barley yellow dwarf virus (BYDV), does not usually occur from singly infected plants. However, this aphid may transmit BYDV-MAV if the plant is doubly infected with this strain and also another, BYDV-RPV. This study was extended to investigate BYDV-MAV transmisson when it occurred together in plants with mixtues of ten other BYDV isolates. Dependent transmission of BYDV-MAV was shown to occur in all instances.

Gill and Chong (1981) investigated the effects of double infection of single cells with two different strains of BYDV using electron microscopy. Infected phloem cells doubly

infected with the two strains were shown to respond differently when compared with their infection with a single strain of the virus. In addition, double infection with the two isolates was shown to predispose the xylem to infection, i.e. there was a breakdown in tissue specificity.

Several aspects of interference and cross-protection have been studied using protoplasts. These are discussed in II.D.8.

D. PROTOPLASTS

1. General

Cocking (1960) first observed the release of protoplasts when he treated tomato root tip cells with fungal cellulase. Gamborg et al. (1978) defined a protoplast as a plant cell possessing a plasma membrane but no cell wall. Since isolated protoplasts are devoid of rigid cellulosic walls, they offer an ideal system for the uptake of particles and macromolecules including plant viruses (Takebe, 1983). The plasma membrane is then the only barrier between the external environment and the cytoplasm (Mantell et al., 1985).

2. Isolation of plant protoplasts

In essence, protoplasts can be isolated by treating plant tissues (leaf, stem or root) with cell wall degrading enzymes. For the study of possible interactions between CMV and MDMV in protoplasts from maize mesophyll tissue, it was important to have some knowledge of the isolation of protoplasts. Since leaf mesophyll tissues of different genotypes vary in their requirements for protoplast release (Mantell et al., 1985), a few basic concepts will briefly be discussed to gain some perspective on this subject.

The ease with which protoplasts can be isolated from a plant, in terms of yield and viability, depends upon a variety of factors. The physical structure of some leaves is such that only a shearing action is required to disrupt the leaf tissue, which can then be treated with cell wall degrading enzymes for the release of protoplasts. In contrast, some leaves have to have the lower epidermis physically removed to expose the mesophyll cells to the enzymes (Mantell et al.,1985). The age of the plant, its physiological condition, the choice of cell-wall degrading enzymes and the choice of osmoticum are variables which have to be investigated for successful protoplast isolation.

Light, humidity, temperature and soil conditions for growth of the plant to be used to isolate protoplasts must be optimized and carefully controlled (Takebe et al., 1968). Most often the youngest fully expanded leaves are taken from well nourished, one- to two-week old plants. Several commercially available cell wall degrading enzymes are available. These are produced from extracts of the fungi Trichoderma and Rhizopus species under various trade names, such as Macerozyme, Pectolyase Y-23, Cellulase 'Onozuka' R10 and Rhozyme. They are used either singly or in combination for protoplast isolation. Ishii and Mogi (1983) reported that the cell wall components of monocotyledonous plants differed from those of dicotyledons. Both pectinase and cellulase were necessary for the isolation of protoplasts from the mesophyll tissue of dicotyledonous species. However, these workers found that cellulase treatment alone would yield protoplasts from gramineaceous plants.

Both epidermal and mesophyll protoplasts are usually generated during the isolation procedure. The latter predominate since epidermal protoplasts are less stable. Protoplasts from epidermal tissue have been used to elucidate the control of direction and rate of spread of viruses which occur in intact leaves (Fannin and Shaw, 1982).

There are two general methods for the isolation of protoplasts. The so-called one- and two-step procedures were devised by Power and Cocking (1969) and Takebe et al.(1968) respectively. As the names imply, these involve either a single (one-step) or a sequential (two-step) treatment with cell wall degrading enzymes. The direct isolation of protoplasts in a single step using a mixture of enzymes is most commonly used as it is a simpler and shorter procedure. To improve protoplast viability and stability, bovine serum albumin is often included in protoplast isolation procedures (Loesch-Fries and Hall, 1980). Takebe (1980) found that protoplast generation was more effective from vacuum-infiltrated leaves. Pre-plasmolysis of leaves by passing them through a series of osmoticums of increasing concentration was in some cases found to be a prerequisite for successful protoplast isolation (Chakraborty, 1973).

3. The isolation of maize protoplasts

As it was of interest to investigate the infection of maize protoplasts with CMV and MDMV, initially a review of methods previously used for the isolation of maize protoplasts had to be made. There have been several reports of using protoplasts isolated from maize leaves for studying physiological and biochemical aspects of maize. Photosynthetic characteristics of chloroplasts such as the C_4-dicarboxylic pathway have been investigated using mesophyll protoplasts (Kanai et al., 1973; Horvath et al., 1978; Day et al., 1981). Respiratory mechanisms and membrane permeability studies have also been facilitated by the use of maize protoplasts (Taylor and Hall, 1976).

Table II.3 summarizes some of the methods previously used for the isolation of maize protoplasts. Both sorbitol and mannitol have been used to maintain an osmotic balance between the maize protoplasts and the surrounding medium. Several different cell wall degrading enzymes appeared to have been successful for protoplast isolation. Cellulysin, cellulase, macerozyme and pectinase have been used together in a variety of combinations.

Once protoplasts have been generated from maize leaves, it is important to keep them in an intact and viable state for as long as possible. This is achieved by suspending them

Table II.3: Methods of isolation of maize mesophyll protoplasts

Maize line	Sterilization	Enzymes used	Osmoticum	Time of incubation	Reference
Punjab Local	2-3 mins 70% ethanol 3 washes sterile H_2O	0,5% pectinase 1% driselase	0,5 M sorbitol 5 mM $CaCl_2.2H_2O$ 1 mM $CaH_4(PO_4)_2$	5-6 hrs at 22°C	Brar et al., 1980
cv. DS 606A	-	0,2% Macerozyme 2% cellulase 0,2% BSA	0,5 M sorbitol 0,2 mM $CaCl_2$ 0,2 mM KH_2PO_4 1 mM $MgCl_2$	2 1/4 hrs at 30°C low light	Day et al., 1981
cv. B73 x Missouri 17	-	2% cellulysin 1% hemicellulase 0,5% pectinase 0,05% BSA	0,6 M mannitol 0,2M $CaCl_2$	2 hrs at 30°C	Lin et al., 1981
Hybrid sweet-corn N65 (Sugar King)	-	2% cellulase	0,6 M sorbitol 20 mM MES buffer 5 mM $MgCl_2$	3-5 hrs at 21-23°C	Kanai and Edwards, 1973

Convar KSC 360	-	0,25% Macerozyme 1,0% cellulase 0,5% K-dextran sulphate	0,6 M sorbitol 0,1 M KH_2PO_4	1,5 hours at 37°C	Horvath et al., 1978
-	0,25% Cetavlon for 1 min, immersion in 70% ethanol for 2 min 3% sodium hypochlorite for 20 mins 6 washes in sterile H_2O	4,5% Meicellase 3,0% cellulase 1500 1% macerozyme 20% Pectinol R10	19% sorbitol	5 hours	Chakraborty, 1973
Golden Bantam	5 mins in 10% ethanol, 1% Zephiran Two washes in 0,6 M sorbitol 10 mM $CaCl_2$	2% cellulysin 1% Hemicellulase 0,5% Macerozyme	0,6 M sorbitol	3 hours at 23°C	Chin and Scott, 1979
cv. Kelvedon 33	10% (w/v) sodium hypochlorite and 0,05% Teepol for 10 mins. 3 washes in sterile H_2O	2,5% macerozyme 5% cellulase	0,6 or 0,8 M sorbitol	15-19 hours at 22°C	Taylor and Hall, 1976
cv. Golden	-	1% cellulase	0,6 M mannitol	2,5-3 hours at 20-25°C	Okuno and Furusawa, 1977

in an incubation medium which contains the osmoticum as well as inorganic nutrients (Okuno and Furusawa, 1977). To reduce bacterial and fungal contamination chloramphenicol, nystatin, carbenicillin and cephaloridine are often included in the incubation medium. It has been noted however that even with these present, the medium may become turbid due to contamination after 48 hours of incubation (Taylor and Hall, 1976).

4. Infection of protoplasts with plant viruses

Cocking (1966) observed the uptake of TMV by tomato fruit protoplasts. Since then many plant protoplast systems have been used to study replication of plant viruses. There are four clear advantages to the use of plant protoplasts for the study of viral replication:

a) Protoplasts represent a relatively homogenous population of cells and virus replication can be essentially synchronous,

b) The possibility of secondary infection by progeny virus is negligible,

c) Easy disruption of protoplasts permits separation of virus particles, proteins, nucleic acids and their analysis,

d) Since each protoplast is in direct contact with the surrounding medium, uptake of radiolabelled precursors (usually amino acids) is facilitated.

It would be unnecessary to discuss in full the contribution which protoplast isolation has made to plant biotechnology. For clarity a general overview of methods of infection will be given. No literature was available on the infection of maize protoplasts with plant viruses. An investigation of CMV and MDMV infection of maize protoplasts was to be done and so previous reports of infection of protoplasts of other plant species by these two viruses were reviewed.

Several factors are known to influence the efficiency of the infection process, and these will be discussed. Protoplasts have been doubly infected with two unrelated viruses or with different strains of a particular virus. Some aspects of double infection of protoplasts will be reviewed.

a) General methods of infection

In most reports, one of two methods appears to be used for the inoculation of protoplasts. Most commonly the so-called 'indirect' method is used. This entails the suspension of protoplasts in one volume of incubation medium, followed by an equal volume of 'inoculation medium' containing the virus particles (Okuno et al., 1977). Alternatively, the protoplast pellet is resuspended directly in the inoculation medium - the 'direct method' (Motoyoshi et al., 1975).

b) Factors influencing inoculation of protoplasts

i) Buffers. The optimum pH and the best buffer to use depend entirely on the virus concerned and on the source of protoplasts. In general, phosphate or citrate buffers are used for inoculation. However the interaction of ionic strength, the chemical nature and the pH of the buffer in relation to the efficiency of infection is not fully understood (Takebe, 1977).

ii) Polycations. Takebe and Otsuki (1969) found that by introducing a polycation such as poly-L-ornithine (PLO) to the inoculum, there was an enhanced efficiency of BMV infection of barley protoplasts. Although the exact mode of action of PLO is not fully understood, it has been established that it is necessary for viruses which have an acidic isoelectric point, e.g. tobacco mosaic virus (TMV), whereas it is not required for those with little negative charge at the pH of the inoculum, e.g. brome mosaic virus (BMV) (Okuno and Furusawa, 1978). At a low pH, BMV carries a net positive charge and under these conditions, PLO was not esential for infection. However at higher pH's, when the virus is negatively charged, PLO must be present for successful inoculation.

It has been suggested that the polycation forms complexes with the virus particles, thus neutralizing their charge and thereby facilitating adsorption to the negatively charged protoplast surface (Mayo and Roberts, 1979). Burgess and co-workers (1973) and Kassanis et al. (1977) proposed, after doing scanning electron microscopy of the entry of TMV into tobacco protoplasts, that lesions are formed in the plasmalemma as a result of the virus/PLO complexes. It is believed that the virus particles enter the plant cell through these lesions. Pinocytosis has also been suggested as a mechanism by which virus infection takes place, although there is little support for this idea (Otsuki et al., 1972).

The molecular weight of the polycation is important and has been found to influence the efficiency of infection (Kassanis et al., 1977). Beier and Breuning (1975) reported that PLO batches are not homogenous with respect to their molecular weights and that they vary in their effectiveness.

iii) Polyethylene glycol. Polyethylene glycol (PEG) is an agent often used for the fusion of cells, and has been found to enhance the entry of certain viruses into protoplasts (Cassells and Cocker, 1980). Again the mode of action of PEG is not fully understood. When PEG is used in the

inoculation procedure, larger amounts of virus are required to achieve infection of an equal level as in the PLO method. High concentrations of PEG can lead to a decrease in the viability of the protoplasts (Maule et al., 1980a).

iv) Osmotic pessure and temperature. It has been reported that osmotic shock, induced by changing the concentration of the osmoticum in the inoculum, can enhance infection efficiencies. The structure and properties of the protoplast membrane undergo changes when subjected to osmotic shock, facilitating virus adsorption and uptake (Okuno and Furusawa, 1978).

The temperature of inoculation is in most cases 25°C, although there have been reports of inoculation at 0°C. At 0°C there is believed to be a change in the fluidity of the membrane which allows for virus entry (Maule et al., 1980b).

v) Protoplast concentration. Mayo (1978) found that for infection of tobacco protoplasts with turnip rosette virus (TRV), a concentration of 0.5 - 5 x 10^5 protoplasts/ml of inoculation medium was optimal for efficient infection. An inverse relationship apparently existed between the percentage of infected protoplasts and the protoplast concentration. It appears that the optimum concentration of protoplasts depends upon the nature of the virus under investigation.

vi) Calcium chloride. Mesophyll protoplasts' membranes may in some cases be stabilized by washing the protoplasts with osmoticum containing calcium chloride (Okuno and Furusawa,1978). Conversely, high concentrations of $CaCl_2$ have been found to have adverse effects on protoplasts of some plants. Albas and Bol (1977) found that 10 mM $CaCl_2$ caused cowpea protoplasts to clump together. In addition Okuno and Furusawa (1978) found that washing barley protoplasts in medium containing $CaCl_2$ after inoculation with BMV, increased the efficiency of infection. Once again the merits of $CaCl_2$ depend on the virus/protoplast system involved.

vii) Concentration of virus in the inoculum. Various concentrations of virus in the inoculum have been reported. If PLO is absent from the system and is not necessary for infection to occur, infection efficiencies have been shown to increase to a certain level with increasing virus concentrations, whereupon further increase in virus concentration has no effect on the infection of the protoplasts. If PLO is necessary for infection, increase in the inoculum concentration causes a parallel increase in efficiency of infection (Sander and Mertes, 1984). After an optimum concentration is reached, however, further increase in virus concentration is detrimental to infection of protoplasts. Albas and Bol (1977) suggested that this occurs becaue high virus concentrations in the inoculum require more PLO which may have a toxic effect on the protoplasts.

5. Methods of detecting viral replication in protoplasts

Once protoplasts have been inoculated with plant viruses, it is essential to be able to detect them and quantitate the course of the infection process at different time intervals. The following methods have been used for this purpose:

a) ELISA - By using the enzyme-linked immunosorbent assay of Clark and Adams (1977), as little as 1 ng of virus/ml may be detected from protoplast extracts (Mayo and Barker, 1983). Thus quantitative estimations of virus concentration in protoplasts may be obtained by this method.

b) Infectivity - Infected protoplasts may be homogenized and then inoculated onto local lesion hosts. In this way, by comparison with an inoculum of known concentration of virus, the virus concentration in the protoplasts may be calculated. This bioassay is the only method available to detect the biological activity of the virus and not merely the presence of virus particles, either complete or degraded.

c) Staining Methods - By conjugating antibodies raised against viruses with dyes, protoplasts infected with these viruses may be visualized. Fluorescent dyes are most commonly used; fluorescein isothiocyanate (FITC) being the most popular. The original method for FITC-labelling of infected protoplasts was devised by Okuno and Takebe (1969).

There have been several modifications to the procedure for staining with FITC; the choice of method depending on the type of protoplast and nature of the infecting virus (Okuno et al., 1972; Maule et al., 1980b). Infected protoplasts may be stained with fluorescent labelled gamma globulins prepared against the virus (the direct method). Alternatively the virus particles in the protoplast may be reacted with antisera to the virus and then the latter detected with fluorescent labelled anti-rabbit serum (the indirect method).

The use of rhodamine B, another fluorescent dye with a different emission wavelength from FITC, offers the option when used in combination with the latter, for distinguishing protoplasts infected with two different viruses.

The immunoperoxidase method was developed as an alternative to FITC-antibody staining as it was found to be more specific than the latter (Ben-Sin and Tien Po, 1982). In this case, once fixed and stained, protoplast preparations may be viewed several days after staining. In contrast, fluorescence emitted by FITC fades rapidly after exposure to UV radiation.

6. Synthesis of viral-induced proteins in protoplasts

Protoplasts infected with viruses are ideal for the analysis of viral-induced proteins and the sequence of their production. This is generally achieved by labelling the

protoplast proteins with radioactive amino acids, such as ^{3}H-leucine, ^{35}S-methionine or ^{14}C proteins. Uptake of these compounds is then followed by detecting them by electrophoresis and autoradiography or by assay of acid-precipitable proteins for the presence of radiolabel.

7. Inhibition of host-protein synthesis

Plant proteins produced by the protoplasts often mask the detection of viral proteins. To overcome this, Sakai and Takebe (1974) irradiated tobacco protoplasts with ultraviolet light. The irradiation has to be closely monitored with respect to its duration (dose), the distance of the germicidal lamp from the protoplasts and its intensity. This is optimized for each protoplast system such that host protein synthesis is reduced and viral protein production unaffected (Maekawa et al., 1981). The most unsatisfactory feature of UV irradiation is the uncertainty about its effects on protoplast metabolism. This may not affect the sequence of the protein production, but could influence the relative amounts of viral proteins produced (Sakai et al., 1977).

Actinomycin D has been used as an indirect inhibitor of host protein synthesis in protoplasts by suppressing mRNA synthesis which is mediated by DNA-depenent RNA polymerase

(Aoki and Takebe, 1975). Actinomycin D has in some cases been found to interfere with viral replication within the protoplasts (Rottier et al., 1979; Maekawa et al., 1981). Takanami et al. (1977) found that UV irradiation and actinomycin D had to be used together to suppress host protein synthesis in CMV infected tobacco protoplasts so that CMV proteins could be detected. Takanami and his colleagues found that high concentrations of actinomycin D were inhibitory to CMV infection and multiplicaton in the protoplasts.

8. Infection of protoplasts with CMV and MDMV

a) Cucumber mosaic virus

Protoplasts have often been used to study aspects of CMV infection, replication and protein synthesis. Table II.4. indicates the variations in infection procedures used by different workers to infect protoplasts with CMV. Infection has been successful with CMV-Y, CMV-Q and CMV-W.

The necessity for PLO for infection varies depending on the strain of CMV used and the type of protoplast. PLO was essential for CMV-Y infection of tobacco protoplasts (Okuno & Furusawa, 1973). It was not critical for infection of cowpea protoplasts with the same strain but its presence did enhance infection (Koike et al., 1977). For CMV-W infection of cowpea

Table II.4: Methods used for infection of protoplasts with different strains of cucumber mosaic virus.

Strain of CMV	Protoplast source	Inoculation buffer	Virus concentration	Concentration of PLO	Points to note	Reference
CMV-Y	N. tabacum L. cv. xanthi	0,02 M potassium citrate pH 5,0	2 ug/ml	2 ug/ml M_r 130 000	Virus preincubated for 5 minutes with PLO before mixing with protoplasts	(a)
CMV-Q	Vigna unguiculata (cowpea)	0,025 M potassium phosphate pH 5,6	2 ug/ml	2 ug/ml M_r 122 000	Virus preincubated for 5 minutes with PLO before mixing with protoplasts	(b)
CMV-W	Cucumis sativus L. cv. Ashley	0,025 M potassium phosphate pH 5,7	2 ug/ml	2 ug/ml M_r 122 000	Incubate viruses with PLO for 5 minutes. Add 10 ml virus/PLO.	(c)
CMV-W	Cucumis sativus L. cv. Ashley	0,025 M potassium phosphate pH 5,7	20 ug/ml	2 ug/ml	Protoplast inoculation with virus repeated twice at $0^{o}C$.	(d)

References

a) Otsuki and Takebe (1973)

b) Gonda and Symons (1979)

c) Maule et al. (1980a)

d) Maule et al. (1980b)

protoplasts Maule et al. (1980b) showed that equal concentrations of the virus and PLO in the inoculum was optimal for infection. A non-linear relationship between virus and PLO concentration existed in the cowpea protoplast system. This is thought to occur as a result of the multipartite genome of CMV. PLO was shown to be essential for CMV-Q infection of cowpea protoplasts (Gonda and Symons, 1979).

Maule et al. (1980b) found that inoculation of cowpea protoplasts with CMV at 0°C enhanced virus uptake into the cells. This temperature is believed to change the properties of the protoplast membrane. Repeated inoculation of the protoplasts also increased the levels of infectivity.

Otsuki and Takebe (1973) found that CMV-Y could be detected using fluorescent-labelled antibodies, 12 hours post-inoculation in tobacco protoplasts. Maule et al. (1980b) showed a peak in CMV-W multiplication 24-48 hours after inoculation, whereas Gonda and Symons (1979) found CMV-Q to multiply rapidly between 0 and 32 hours post-infection. Th virus particles were shown by fluorescent-labelled antibodies to accumulate in the cytoplasm and nuclei and not in the chloroplasts (Otsuki and Takebe, 1973; Maule et al., 1980b).

i) Proteins induced by CMV infection of protoplasts

It is often of interest to characterize the in vivo gene products of a virus when it infects protoplasts and to analyse the time course of their translation products.

Gonda and Symons (1979) infected cowpea protoplasts with CMV-Q to investigate the time course of synthesis of CMV particles, CMV coat protein and its four RNAs. Synthesis of the coat protein was determined using ^{3}H-leucine (50 uCi/mM) or ^{14}C protein hydrolysate (20 uCi/mM). These were added to the infected protoplasts immediately after their inoculation so that "continuous" labelling was carried out. Alternatively, the protoplasts were "pulse-labelled". This means that the radioactive label was added to the protoplast supensions at intervals during their incubation. Only the coat protein (M_r= 24,5 kd) of CMV-Q could be detected by continuous labelling. By pulse-labelling it was shown that the CMV coat protein is produced as early as 10 hours after inoculation and reaches a maximum concentration in the protoplasts 15 h post-infection. In contrast, TMV coat protein synthesis increases steadily with time post-infection (Siegel et al., 1978). This could reflect differences in the assembly mechanisms for rod-shaped and spherical viruses (Gonda and Symons, 1979).

Other viral-induced proteins produced by CMV infection of protoplasts could not be detected by Gonda and Symons (1979) using one-dimensional electrophoresis. Other proteins produced at lower levels may have been distinguished more clearly from the host proteins if two-dimensional separation had been carried out.

b) Maize dwarf mosaic virus

No reports of protoplasts infected with MDMV could be found. Xu et al., (1984) infected tobacco protoplasts with tobacco vein mottling virus which, like MDMV, is a potyvirus. The method which they used involved preincubation of 1 ug/ml of the virus with 1 ug/ml of PLO. After inoculation of the tobacco protoplasts, they were washed in medium containing $CaCl_2$. After 60 hours, over 75% of the protoplasts were shown by microscopy to be infected with TVMV.

9. Resistance of protoplasts to virus infection

Investigations of the mechanisms involved in resistance of plants to viruses have been facilitated by the use of plant protoplasts. Complete resistance of a plant to virus infection implies that it is a non-host for that virus. If viral replication is restricted by limiting the spread of the virus, the plant is said to be hypersensitive. The expression of resistance in single cells can be differentiated from a mechanism operating in whole plants by the use of protoplasts.

Otsuki *et al*. (1972) found that TMV replicated in both protoplasts with the N gene for hypersensitivity and in susceptible protoplasts. Koike *et al*. (1977) and Gonda and Symons (1979) showed that CMV which does not normally infect cowpea plants will infect protoplasts isolated from these plants. Furusawa and Okuno (1978) showed that a strain of BMV would infect protoplasts of the non-host plant *Raphanus sativus*. Maule *et al*. (1980a) investigated the resistance of protoplasts prepared from different cucumber cultivars to CMV infection. Using fluorescent-labelled antibodies they found that there was a difference in the amounts of virus extracted from protoplasts of resistant and susceptible cucumber cultivars. Virus particles were shown to bind equally to the resistant and susceptible protoplasts so that the results were not due to differences in the requirements for inoculation.

10. Double infection of protoplasts

To gain a better understanding at a cellular level of the interaction of unrelated viruses and of two strains of the same virus when they infect the cells simultaneously, protoplasts have been used. (Otsuki and Takebe, 1976; 1978; Barker and Harrison, 1977; 1978; Watts and Dawson, 1980).

For mixed infection of protoplasts, the procedures used are normally similar to those for single infections except that conditions are moderated so that they suit both viruses (Otsuki and Takebe, 1976). Protoplasts may be infected simultaneously i.e. both viruses together in the inoculum, or sequentially i.e. one virus followed after a time interval by another, as was done with brome mosaic virus and cowpea chlorotic mottle virus by Watts and Dawson (1980).

Virus content of doubly infected protoplasts can be estimated by fluorescent labelling of specific antibodies prepared against the viruses. If the percentage of protoplasts infected with one virus (A), the second virus (B) and the total number of infected protoplasts (C) (using mixed anti-A and anti-B serum) is determined, then A + B - C will give the percentage of doubly infected protoplasts (Watts and Dawson, 1980). Cross-reaction of antisera with the reciprocal virus will reduce cross-absorption (Otsuki and Takebe, 1978).

a) Infection of protoplasts with unrelated viruses:

When Otsuki and Takebe (1976) infected tobacco protoplasts with CMV and TMV they found that:

i) CMV multiplication was impeded when TMV had already infected the protoplasts,

ii) When CMV and TMV were inoculated simultaneously the replication of both viruses was unaffected,

iii) When CMV was inoculated first, followed by TMV there was no effect on the replication of TMV.

Barker and Harrison (1977) found that when tobacco protoplasts were doubly infected with raspberry ringspot vius (RRV) and tobacco rattle virus (TRV), aggregates of the RRV particles formed. These do not occur in singly infected protoplasts. It was shown that there was no increase in the concentration of the RRV and no heterologous coating of the two viruses occurred. The reason for the formation of the aggregates is unclear.

Interference was demonstrated when tobacco protoplasts were infected with brome moaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) (Watts and Dawson, 1980). When CCMV was inoculated first and BMV was inoculated after a time interval, there was no infection by BMV. When the time interval was closely monitored it was shown that, as the time before BMV

inoculation was increased, the efficiency of BMV infection decreased until when 8 hours had elapsed between CCMV infection and BMV inoculation, no BMV infection occurred. When the reciprocal test was carried out i.e. BMV first, then CCMV, complete interference was exhibited only after an 18 hour delay between inoculations. The mechanisms of entry of the virus into the protoplasts is thought to differ. This would account for the time differences between the two viruses before the onset of complete interference. When BMV was irradiated with UV, there was no interference in the subsequent infection of CCMV. It was hypothesized that the interference mechanism was connected to the early stages of the infection process and probably involved the RNA of each virus.

b) Double infection of the protoplasts with different strains of virus:

When protoplasts were infected with two strains of TMV, some of the progeny virus particles were shown to contain coat proteins which were a mixture of the coat proteins of the two strains (Otsuki and Takebe, 1978). Barker and Harrison (1978) investigated the interactions of different strains of RRV doubly infecting tobacco protoplasts. They found that two strains of RRV can multiply together in a single cell, but that some interference between strains occurred. When protoplasts were isolated from leaves systemically infected with one strain, superinfection with a second virus could occur.

Other virus-virus and virus-host systems will have to be investigated to gain further insight into their complex mechanisms. Obviously protoplasts are ideal for this type of study.

E. LIPOSOMES

1. General

Bangham and his co-workers (1965) were the first to prepare liposomes as models for the study of membrane properties. When it was realized in 1971 that liposomes could entrap small solute molecules within their structure, it was envisaged that they could possibly be used to deliver drugs, antibodies and other therapeutic compounds to animal cells (Tyrell et al., 1976). Further work on the use of liposomes for the entrapment of antibiotics, cell-modifying compounds and anti-tumour drugs is on-going. The possible application of liposomes in plant protoplast systems was realized and several workers began to investigate the possibility of liposomes delivery of plant viral nucleoproteins and RNA to plant protoplasts (Fukunaga et al., 1981).

2. Liposome structure and physical properties

Tyrell et al. (1976) refer to liposomes as "typical crystal vesicles" since, when phospholipids are suspended in excess of aqueous solution, they spontaneously form multilamellar, concentric vesicles. The balance between the repulsive and attractive forces of the liposome layers determines their separation. Thus the distance between the bilayers, and consequently the volume of entrapped aqueous phase, can be enlarged by increasing the proportion of charged lipid in the phospholipid preparation.

Many phospholipids can be used for the formation of liposomes. Most commonly used are phosphotidylcholine, phosphotidylserine, phosphotidylethanolamine and sterylamine (Szoka et al., 1978).

Although it has been shown that entrapped solutes will leak out of liposomes, large proteins in general will not permeate the bilayer unless the structure of the liposome is disrupted by detergent (Tyrell et al., 1976).

3. Methods of preparing liposomes

Following the realization that liposomes are important for delivery of particles and compounds to cells, several methods for their preparation have been devised.

Multilamellar vesicles (MLV's) were originally made by drying phospholipid under vacuum in a rotary evaporator so that a film of lipid formed, devoid of organic solvent, on the walls of the evaporating flask (Bangham et al., 1965). When the aqueous solution is added, the lipid falls away from the walls, swells and in so doing entraps the aqueous phase in large multilamellar vesicles.

The close apposition of the concentric bilayers of MLV's reduces their capacity for a large internal aqueous space (Szoka et al., 1978). Small unilamellar vesicles (SLV's) have a high surface area:encapsulating ratio so that they, like MLV's, can only entrap a small aqueous volume. To overcome this problem, Batziri and Korn (1975) attempted to inject a liposome preparation suspended in ethanol into the aqueous phase using a fine needle. This was not successful. If ether was inoculated, however, large unilamellar vesicles (LUV's) could be formed. Although these have a large encapsulating volume, they have a low efficiency of entrapment (Tyrell et al., 1976).

Szoka and Papahadjopoulos (1978) devised a method for preparation of liposomes by "reverse phase evaporation". These liposomes possessed the following properties:

a) The ability to entrap a large percentage of the aqueous phase,

b) A high aqueous space:lipid ratio,

c) Could be made from a wide range of lipids.

Their method consisted of removal of the solvent from the lipid mixture by rotary evaporation, followed by redissociation in an organic phase, such as diethyl or isopropyl ether. "Inverted micelles" are thus formed. The aqueous phase is then added under nitrogen gas, followed by sonication. The latter apparently causes small water droplets to form, which are stabilized by the phospholipid monolayer. The inverted micelles collapse as the organic phase is removed by evaporation. A viscous gel forms which also collapses under further evaporation. Excess lipid forms a complete bilayer around the micelles to form vesicles some 200-500 nm in diameter depending on the lipid composition, the solvent used, and the relative amounts of aqueous phase, organic solvent and phospholipid.

More recently liposomes have been produced using a dehydration/rehydration method (Kirby and Gregoriadis, 1984). By this method the harmful effects of sonication and exposure to organic solvents are avoided. REV's are prepared as described by Szoka and Papahadjopoulous (1978), flash frozen and then lyophilized by freeze drying. This constitutes the dehydration part of the method. Rehydration occurs when the aqueous solution to be entrapped is added. This induces fusion of the performed liposomes. The resultant vesicles are approximately 0,3 u in diameter and are mainly multilamellar. The simplicity of the procedure, the ability to store the lyophilized preparation for indefinite lengths of time and the mildness of the method are great advantages. Their heterogenicity in size and their relatively small encapsulation volume are their main limitations.

It is obviously important to remove all non-associated solute from the liposome preparation. In general, gel filtration, centrifugation and dialysis are the methods used to achieve this. The choice of method is dependent on the nature of the liposomes and their entrapped solute (Fraley et al., 1982).

In order to observe the activity of the encapsulated compound, solubilization of the liposome preparation by addition of a detergent [such as Triton X-100 (1% v/v)] must be carried out (Tyrell et al., 1976; Lurquin, 1979).

4. Application of liposomes to plant protoplast systems

Since liposomes had been used as carriers of biologically active materials to animal cells, there seemed no apparent reason why they could not be used in plant protoplast research. Cassells (1978) demonstrated that positively charged liposomes could sequester a fluorescent dye, fluorescein diacetate, and then be incubated with protoplasts. After a few hours the protoplasts were shown to be fluorescing, indicating that the contents of the liposomes had been transferred to the plant cells.

Lurquin (1979) incorporated the plasmid pBR322 into liposomes, fused these to cowpea protoplasts and investigated the incorporation of the plasmid DNA into the host genome.

Liposome encapsulation of the DNA protects it from degradation by DNases. The binding of the liposomes containing the DNA was visualized by staining with DAPI, a fluorescent dye, which complexes with DNA. The donor DNA was shown to be transferred to the nuclei in an undegraded form. The functional activity and whether the DNA would be integrated were not investigated. Subsequent to this work, there have been numerous reports of liposome-mediated transfer of viral nucleoproteins and RNA to plant protoplasts. The efficiency of infection of RNA in particular has been shown to be greatly improved when encapsulated in liposomes. The lipid vesicles protect the nucleic acid from degradation and exhibit a low toxicity towards the plant cells (Fraley et al., 1982; Fraley, 1983).

It appears from the literature that the type of liposomes used for fusion to plant protoplasts vary with respect to their composition and therefore their charge. Conditions for fusion seem to be optimized for the particular virus-RNA under study.

Initially TMV-RNA was encapsulated in liposomes (LUV's) and fused with tobacco protoplasts from suspension culture by a PEG-mediated interaction. Only 3% of the protoplasts could be infected with free RNA whereas there was a 50% efficiency of infection using liposome-encapsulated RNA (Fukunaga et al., 1981). Nagata et al. (1981) used REV's for

encapsulation of TMV-RNA. The advantage of REV's is that they can be prepared with different surface properties depending on the type of lipid used. Those liposomes with a net positive charge would have a greater affinity for the negatively charged protoplasts. Fraley et al. (1982), Watanabe et al. (1982) and Rouze et al. (1983) also investigated the infection of tobacco protoplasts, from both mesophyll and suspension cultures, by TMV-RNA encapsulated in liposomes.

Rollo and Hull (1982) experienced difficulties in fusing REV's containing turnip rosette virus (TRV) to protoplasts. There are several factors to be considered when fusing liposomes to protoplasts:

a) Polyethylene glycol (PEG) and other polycations appear to be impotant in the liposome-protoplast interaction. The mechanism by which PEG mediates the intracellular delivery of the liposomes' contents is not clear (Fraley et al., 1982). Fusion has been suggested to occur or alternatively an endocytosis-like process could take place. The optimum time of incubation with PEG seems to be dependent on the protoplast system being used; whether suspension cultured cells or mesophyll cells (Fraley, 1983).

b) Calcium ions in the washing procedure after the liposome-protoplast interaction are in some cases necessary. They are believed to neuralize the charges on either the liposomes or protoplasts, thereby facilitating association between the two (Rouze et al., 1983).

c) Temperature of incubation may also have either a deleterious or enhancing effect on the frequency of infection (Fukunaga et al., 1981).

5. Assays for liposome-mediated infection

It is obviously necessary to determine whether the contents of the liposomes which are transferred to the protoplasts are indeed biologically active. It is often difficult to distinguish between vesicles containing radio-labelled or fluorescent-labelled compounds which have merely associated with the surface of the protoplasts, or have delivered their contents intracellularly. In addition, leakage from the liposomes may occur and apparent uptake by damaged cells may cause confusion in the results obtained (Tyrell et al., 1976; Fraley, 1983). As mentioned previously, unencapsulated material is removed from the liposome preparation by gradient centrifugation, gel filtration or dialysis before fusion with the protoplasts (Fraley et al., 1982).

In general, assay techniques similar to those used in free virus/RNA uptake by protoplasts are carried out to ascertain how effective liposome-mediated transfer has been. Fluorescent labelling, ELISA, incorporation of radiolabel by scintillation counting and infectivity tests are used (Fukunaga et al., 1981; Nagata et al., 1981; Fraley et al., 1982; Rouze et al., 1983).

6. Future prospects

Lurquin (1979) successfully introduced foreign plasmid DNA into cowpea protoplasts using liposomes in the presence of PEG. The implications of this are far-reaching in terms of genetic manipulation and breeding of plants, particularly agriculturally important crops. New approaches to this aspect include introduction of defined genes mediated by chromosome transfer, microinjection of DNA into cells, uptake of DNA directly into protoplasts and gene transfer using genetically modified bacteria such as *Agrobacterium* or viruses such as cauliflower mosaic virus. Considerable research efforts are being channelled into this avenue of research (Lorz *et al.*, 1984; 1985).

CHAPTER III

INVESTIGATION OF NATURAL FIELD INFECTED MAIZE

Investigations of natural field infected maize plants showed that double infections appear to be a common phenomenon. Three separate examples, originating in different areas of South Africa were examined carefully to establish the identity of the viruses in the mixed infections. These will be discussed further.

A. 'McARTHUR' MAIZE

On a visit to Natal, Professor von Wechmar was given a specimen of a maize plant which originated in a field in the district of Dundee. It had been assumed that the severe yellowing and stunting of the plant were the result of chemical fumes released from industrial chimneys in the vicinity. The yellowing and stunting reminded Professor von Wechmar of viral infection; however the symptoms did not appear to be those of a single infection of viruses known to occur in maize in South Africa i.e. MDMV or CMV (Chauhan, 1985; Lupuwana, 1985). From this it was hypothesised that more than one infectious agent was involved.

The infectious agents were apparently easily sap transmissible to maize and initially caused the normal mosaic associated with MDMV infection. Two weeks later the infected plants started to turn yellow with a distinct red mottle/mosaic pattern developing on older leaves. This is not characteristic of a MDMV infection only. Inoculated cucumber, tobacco, zinnia and Chenopodium quinoa plants also developed uncharacterstic symptoms, mainly chlorosis and severe dwarfing, indicating that another virus was present (von Wechmar, Progress Report, Maize Virus Research 1984/1985; A Milligan, Honours Project, 1984).

Evidence for a double infection was obtained by:

(1) Electron microscopic investigation: Leaf-dip preparations stained with uranyl acetate revealed both filamentous and spherical particles (Figure III.1). The spherical particles were mostly degraded.

(2) DAS-ELISA (IX.D.5): Concentrated maize extracts were prepared from sap-inoculated plants and tested for six different isolates of CMV. Details of the specific anti-CMV sera used and the results are presented in Table III.1

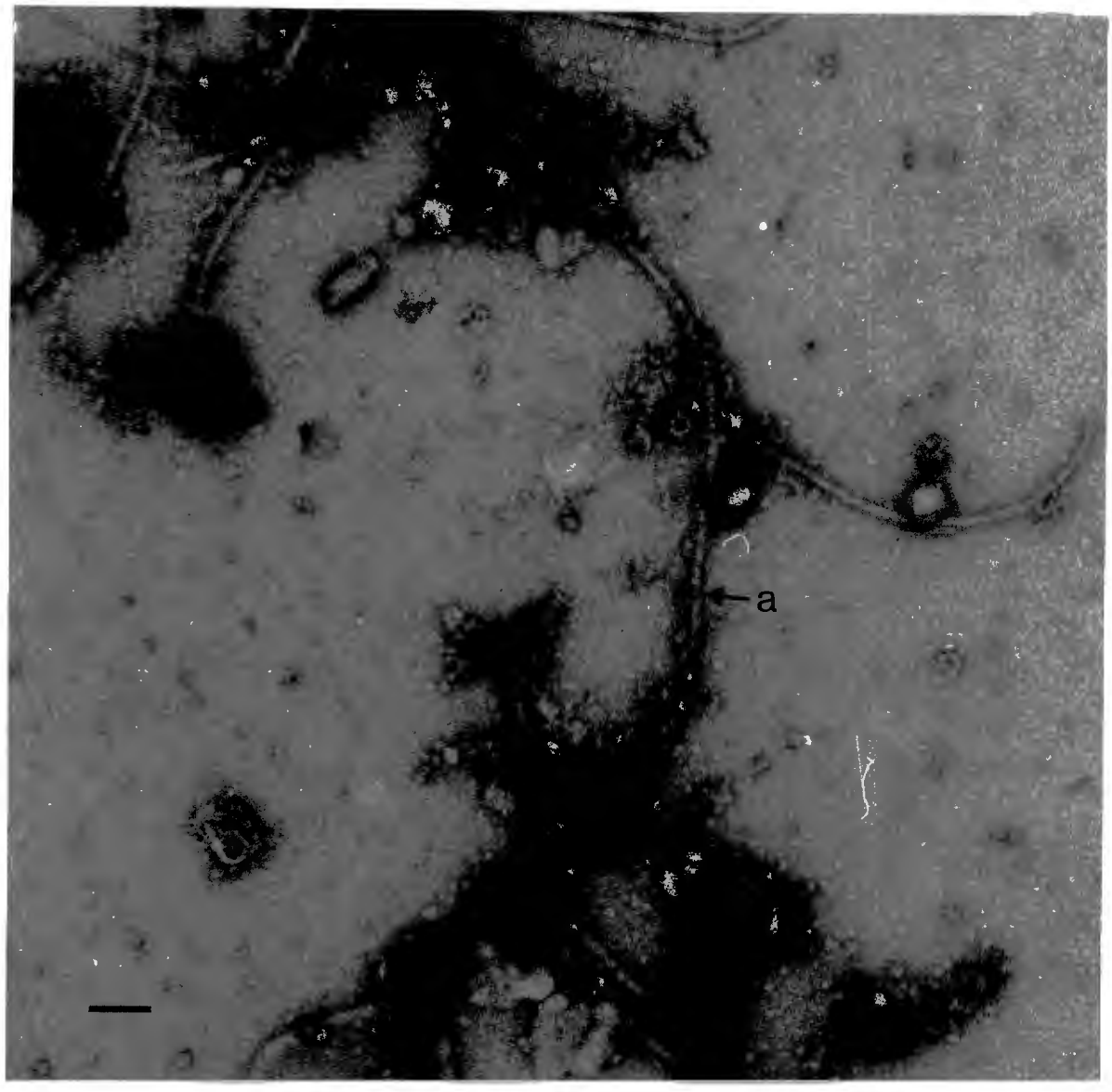


Figure III.1: Electron micrograph of leaf-dip preparation of a natural mixed infection in maize of cucumber mosaic virus and maize dwarf mosaic virus ('McArthur' maize). The specimen was stained with uranyl acetate and the magnification is 90 000 x. Bar = 100 nm. Note the presence of filamentous particles (a), and spherical particles (b), most of which are dissociated. Serological tests identified the filaments as MDMV-B and the spherical particles as CMV. See also Table III.1 and Figure III.2.

Table III.1: Results of DAS-ELISA using antiserum prepared against different strains of CMV and a preparation of 'McArthur' maize purified from sap-inoculated maize.

Antiserum[1]	As-dilution[2]	Absorbance at 405 nm[5]		
		Control[4]	Homologous CMV strain	'McArthur' isolate
anti-CMV-Q[3]	1/250	0,042	0,356	0,056
anti-CMV-Y[3]	1/400	0,056	N/D[6]	0,083
anti-CMV-Is	1/400	0,092	1,351	0,769
anti-CMV-K	1/250	0,104	1,035	0,735
anti-CMV-Tob	1/400	0,096	1,284	0,684
anti-CMV-S	1/400	0,054	N/D	0,186

1. See IX.B.
2. Antisera dilution IgG and conjugate, in antibody dilution buffer (IX.A.3(a)) and post-coating buffer (IX.A.3.(c)) respectively.
3. The antisera were early bleedings which could explain the negative results.
4. Control: Uninfected maize
5. Positive reactions have $OD_{405nm} > 0,2$
6. N/D-Not done

It is evident that the 'McArthur'-isolate contained antigens which reacted strongly positive with antisera raised against CMV-Is, CMV-K and CMV-Tob. The fact that anti-CMV-Q, -Y and -S are negative could be attributed to their being early bleedings.

(3) Enzyme immunoelectroblotting tests. (IEB) (For details of method see IX.D.7). Immunoblotting confirmed results obtained earlier by DAS-ELISA and electron microscopy, namely that two viruses were present. Figure III, 2.a. shows clearly that anti-CMV-Tob serum recognizes a 24,5 kd protein in the 'McArthur' preparation corresponding to that of purified CMV-Tob (lane 3). In addition proteins of higher molecular weight were recognised. The 'McArthur'-isolate was probed using anti-MDMV-B-ST serum. The presence of the 37 kd MDMV protein and the characteristic profile of MDMV polypeptides (Chauhan, 1985) are clearly evident (Figure III.2. b). Antiserum prepared against the 'McArthur'-isolate (IX.D.4.) was also used in this assay (Figure III.2.c). This antiserum recognized both the MDMV and CMV proteins in purified preparations of these two viruses. Thus the antiserum prepared against the unfractionated preparation of 'McArthur' maize stimulated the production of antibodies against both infectious agents. The advantage of using the original preparation for

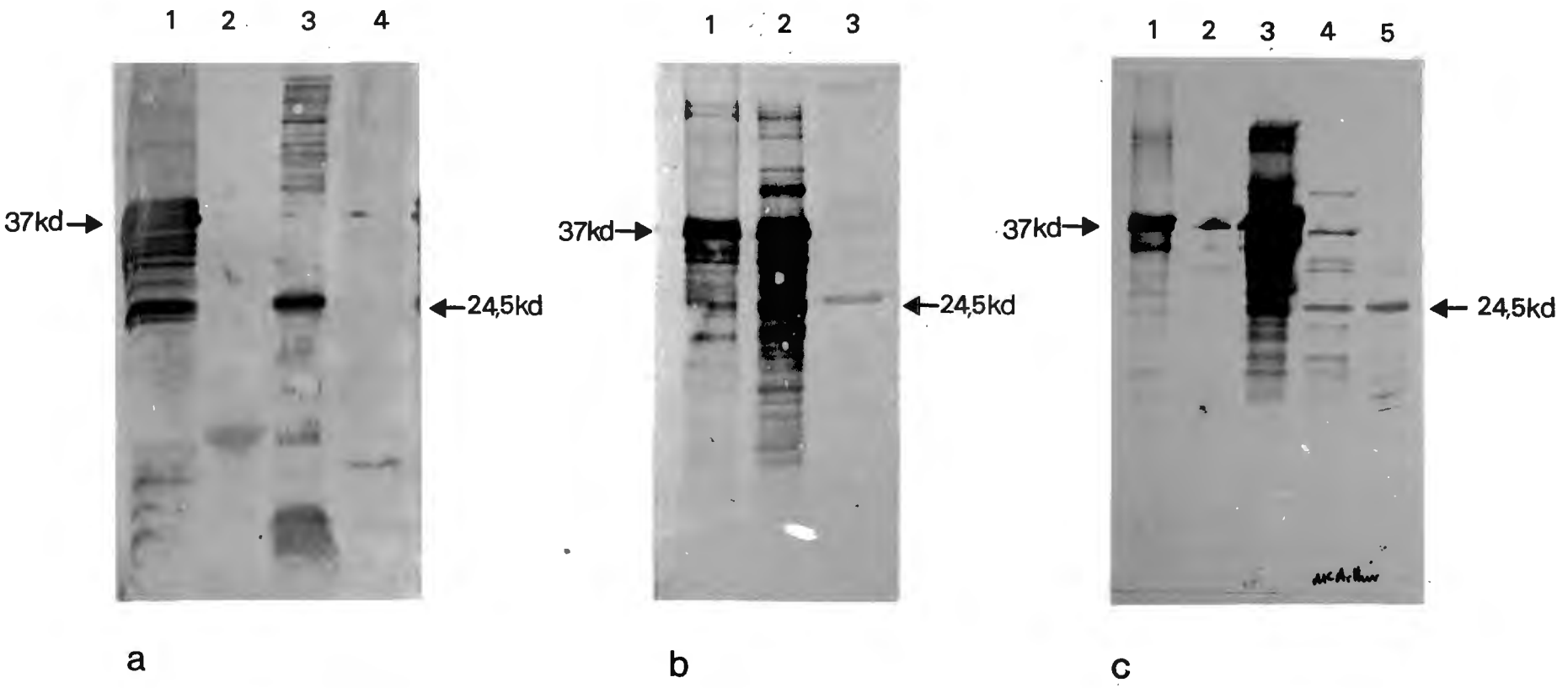

1 2 3 4
37kd
24,5kd
a
1 2 3
37kd
24,5kd
b
1 2 3 4 5
37kd
24,5kd
c

Figure III. 2.a, b, c. Immunoelectroblot test of 'McArthur' maize-isolate probed with different antisera. The 'McArthur' maize was extracted for CMV (IX.D.3(a))[1]:

(a) Probed with anti-CMV-Tob serum at a 1/40 dilution. The characteristic 24,5 kd protein is evident (Lupuwana, 1985).

Lane 1 - 'McArthur'-isolate concentrated extract.
2 - BMV-standard
3 - CMV-Tob standard
4 - Uninfected maize

(b) Probed with anti-MDMV-B-ST serum at a 1/30 dilution. The 37 kd protein in the 'McArthur' extract corresponds to that of MDMV-B-ST (Chauhan, 1985).

Lane 1 - MDMV-B-ST standard
2 - 'McArthur'-isolate
3 - CMV-Tob standard

(c) Probed with anti-'McArthur' serum at a 1/40 dilution. The antiserum recognises the 37 kd and 24,5 kd proteins of MDMV and CMV respectively.

Lane 1 - MDMV-B-ST standard
2 - Protein M_r marker
3 - 'McArthur'-isolate
4 - CMV-K standard
5 - CMV-Tob standard.

1. Extracted for CMV to preserve the unstable virus particles. If extracted for MDMV-B (which was also present) the CMV would be lost in the process.

immunization is that the second component was not lost, as may have occurred if it been selectively purified. This must be taken into consideration when applying Koch's postulate. It may not be possible to initiate the original disease with only a single agent.

Concluding remark: The results obtained from these limited laboratory tests showed that two viruses were involved i.e. maize dwarf mosaic virus strain-B and a strain of cucumber mosaic virus .

B. 'ROODEPLAAT B' MAIZE

In January 1984, Miss Ramola Chauhan collected two maize samples at the Roodeplaat Horticultural Institute exhibiting symptoms of unusual virus infection, not typical of a single infection caused by known viruses occurring in South Africa. These isolates were identified as 'Roodeplaat A' and 'Roodeplaat B'. Miss Chauhan investigated the former isolate (Chauhan, 1985).

Figure III.3 shows the yellowing, mosaic symptoms expressed by the 'Roodeplaat B' sample after the first sap transmission to maize. Sap-inoculation to maize seedlings (cv. KEP) gave rise to mosaic symptoms in approximately 60% of the seedlings after 7 days. The 'Roodeplaat B'-isolate was examined in a similar manner as the 'McArthur'-isolate (III.A).

Figure III.3: Strong yellow mosaic and distinct striping characterized the symptoms of the 'Roodeplaat B' isolate after sap transmission to maize seedlings. This symptom is not characteristic for a single infection.

(1) Electron microscopic investigation showed that a filamentous and a CMV-like particle were present in the 'Roodeplaat B'-isolate (Figure III.4).

(2) DAS-ELISA tests (IX.D.5) with extracts of 'Roodeplaat' B from sap-inoculated maize (cv. KEP) (CMV-method,IX.D.3.(a)) gave positive reactions with anti-CMV-Tob and anti-MDMV-B-ST sera (Table III.2)

Table III.2: Results of DAS-ELISA with 'Roodeplaat B' maize-extract against anti-MDMV-B-ST and anti-CMV-Tob sera.

Antigen[1]	Dilution	Absorbance at 405 nm[2]	
		Anti-MDMV-B-ST[3]	anti-CMV-Tob[4]
Control[5]	1/4	0,035	0,012
MDMV-B-ST standard	1/100	0,485	0,032
Roodeplaat B maize	1/4	0,376	0,207
CMV-Tob standard	1/1000	0,020	0,689

1. Antigen was a 10-fold concentrated plant extract and was diluted in post-coating buffer (IX.A.3.(c)).
2. Positive reactions have an absorbance at 405 nm > 0,08 which is double the reading obtained for uninfected maize.
3. Anti-MDMV-B-ST IgG and conjugate used at 1/500 dilution.
4. Anti-CMV-Tob IgG and conjugate used at 1/400 dilution.
5. Control: uninfected maize.

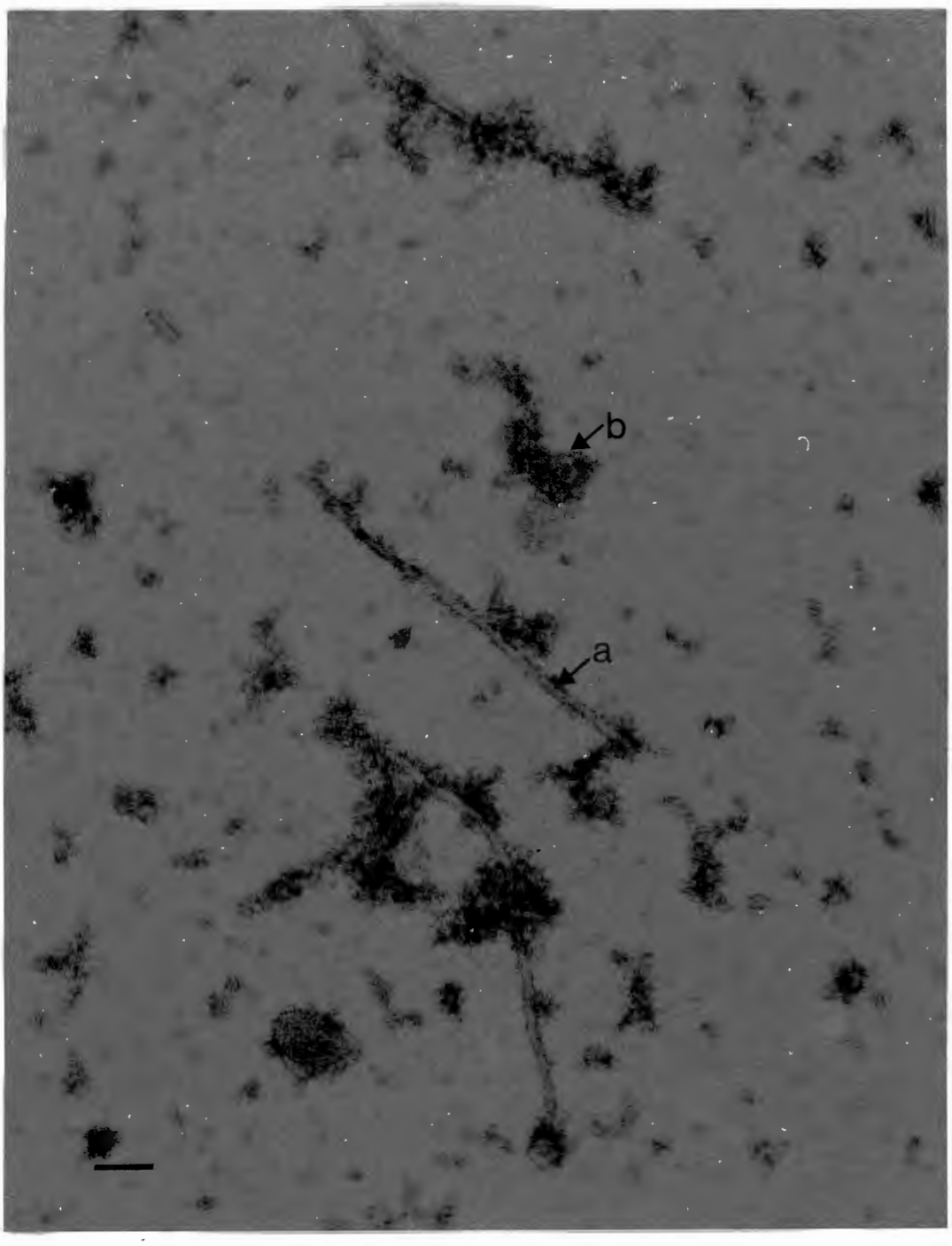


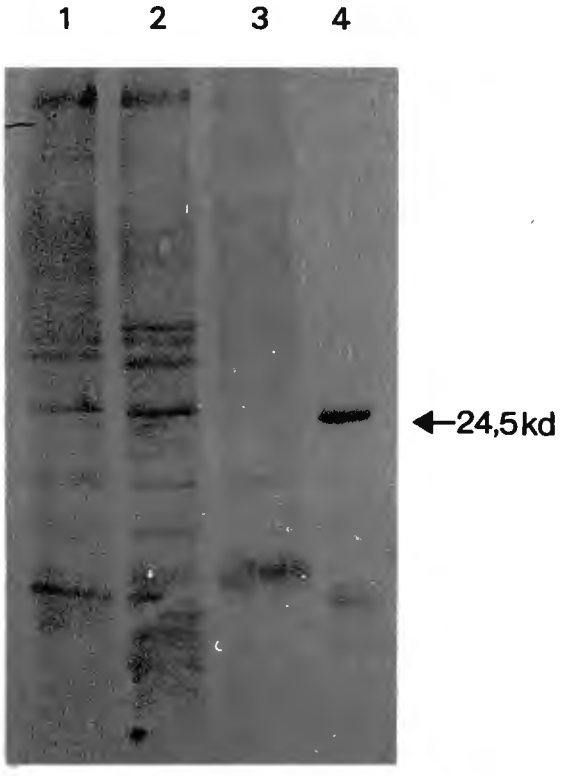
Figure III.4: Electron micrograph of a leaf-dip preparation of 'Roodeplaat B' isolate stained with uranyl acetate. Magnification = 90 000. The bar represents 100 nm. The presence of both filamentous (a), and spherical (b) particles was evident. These were shown to react positively with anti-MDMV-B-ST and anti-CMV-Tob sera (see Table III.2).

(3) Results of the enzyme immunoelectroblot test confirmed those obtained by DAS-ELISA. Anti-CMV-Tob serum probed the 24,5 kd protein corresponding to that of purified CMV-K (Figure III.5.a, lane 2). In addition antiserum prepared against MDMV-B-ST recognised the 37 kd protein profile of this virus in the 'Roodeplaat B'- isolate (Figure III.5.b, lane 2).

C. HENTIE MAIZE

In January 1984, Professor von Wechmar collected infected maize plants on the farm of Mr Hentie Groenewald near Potchefstroom exhibiting symptoms typical for MDMV-B infection (Figure III.6.b).

Figure III.6.a illustrates a portion of the field and clearly shows the open spaces where maize seed either did not germinate and/or seedlings died early. As the majority of the remaining plants in this field were virus-infected, the first assumption is the more probable one (von Wechmar and Chauhan, 1984; Chauhan, 1985).



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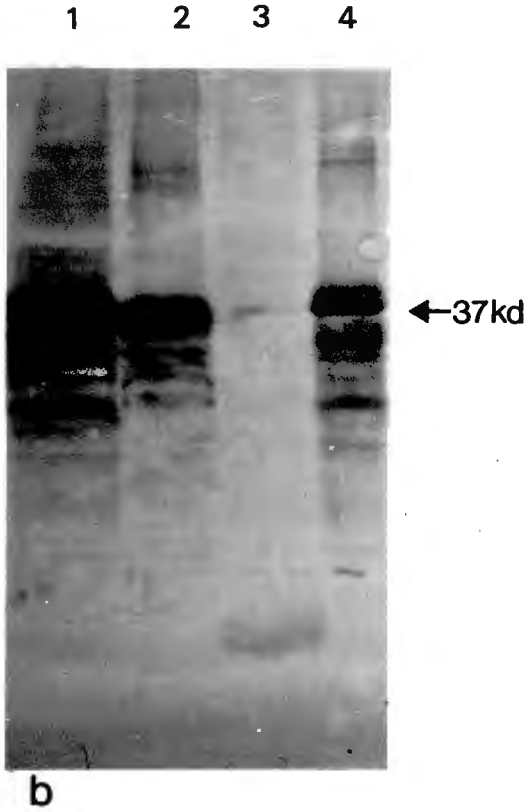

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Figure III.5a and b: Immunoelectroblot tests of 'Roodeplaat B' maize. The isolate was extracted for CMV (IX.D.3(a)).

(a) Blot probed with anti-CMV-Tob serum at a 1/30 dilution. The 24,5 kd protein of CMV is recognised (lane 2).

Lane 1 - Maize seedlings showing abnormal symptoms (seed-transmitted CMV; Knox, 1983)

2 - 'Roodeplaat B'-isolate

3 - BMV standard

4 - CMV-K standard

(b) Blot probed with anti-MDMV-B serum at 1/30 dilution. The 37 kd protein corresponding to that of the MDMV-B-ST standard is evident in the extract (lane 2).

Lane 1 - 'Hentie'-isolate

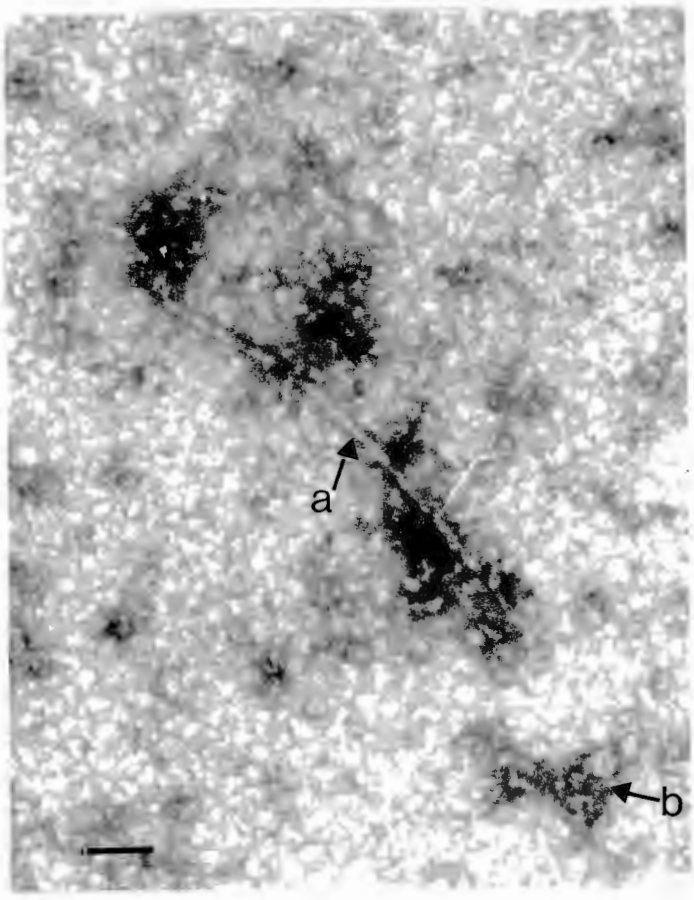
2 - 'Roodeplaat B'-isolate

3 - Uninfected maize

4 - MDMV-B-ST standard

a

b



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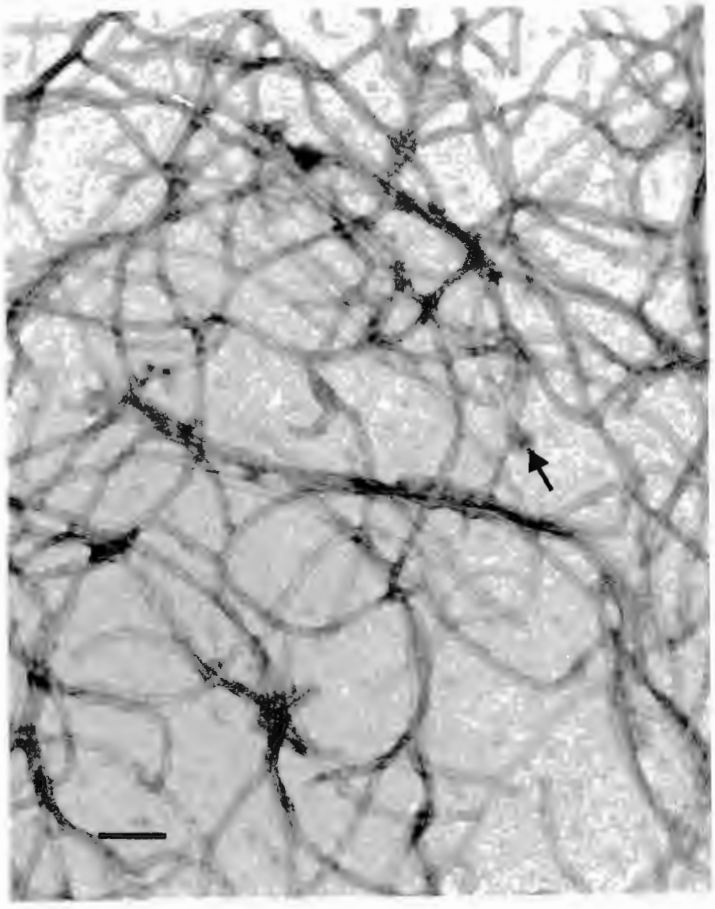

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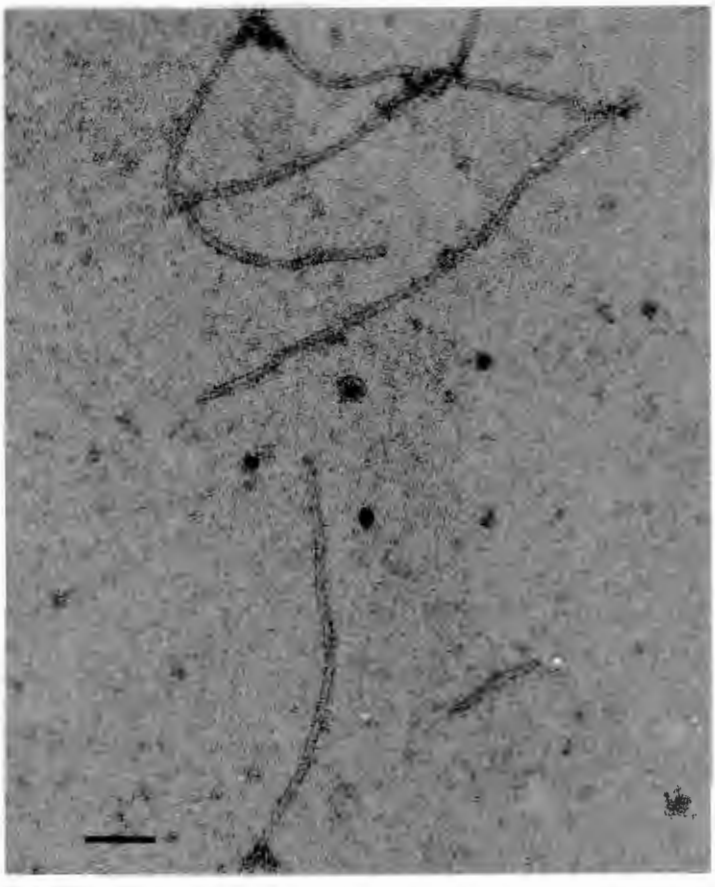

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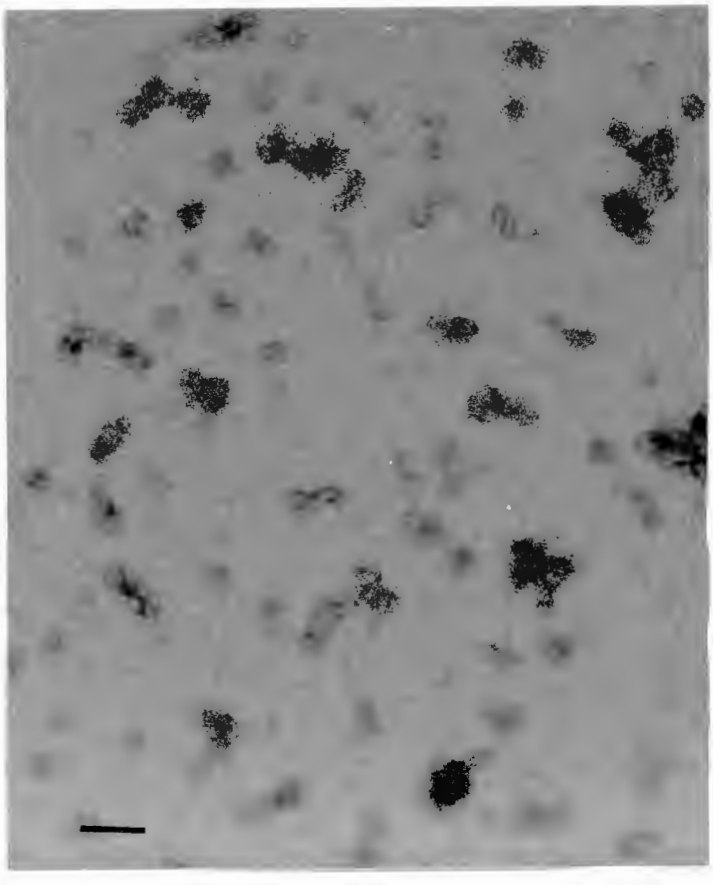


d

Figure III.7 a, b, c and d: Electron micrographs of the 'Hentie'-isolate. Magnification 90 000 x. Bar = 100 nm.

(a) Leaf-dip preparations of the original field collected 'Hentie'-isolate. Uranyl acetate was used for staining. Filamentous (a) and degraded spherical particles (b) are evident.

(b) High concentrations of filamentous particles were isolated when the virus was propagated on maize. Arrows indicate spherical particles which occurred at a lower concentration when propagated in maize. Uranyl acetate was used for staining.

(c) Immuno-electron micrograph of a leaf-dip preparation of the original field collected plant. Anti-MDMV-B serum trapped filamentous particles and some spherical particles (see text).

(d) Anti-CMV-Is serum trapped degraded icosahedral particles from a preparation of N. clevelandii tobacco plants sap-inoculated with the 'Hentie'-isolate.

Table III.3: Results of DAS-ELISA of 'Hentie'-isolate propagated on various hosts using several anti-CMV sera.

Antisera	Dilution[1]	Absorbance at 405 nm[3] of Hentie isolates[2] propagated on different hosts				
		Uninfected	Squash	maize	N. glutinosa	N. clevelandii
anti-CMV-Y[4]	1/400	0,056	0,141	0,246	0,180	0,166
anti-CMV-Is	1/400	0,092	1,076	0,254	0,249	0,547
anti-CMV-K	1/250	0,104	0,378	1,086	0,402	0,305
anti-CMV-S	1/400	0,054	0,390	0,612	0,415	0,369
anti-CMV-Q[4]	1/250	0,042	0,057	0,086	0,042	0,047
anti-CMV-Tob	1/400	0,096	0,066	0,071	0,057	0,112

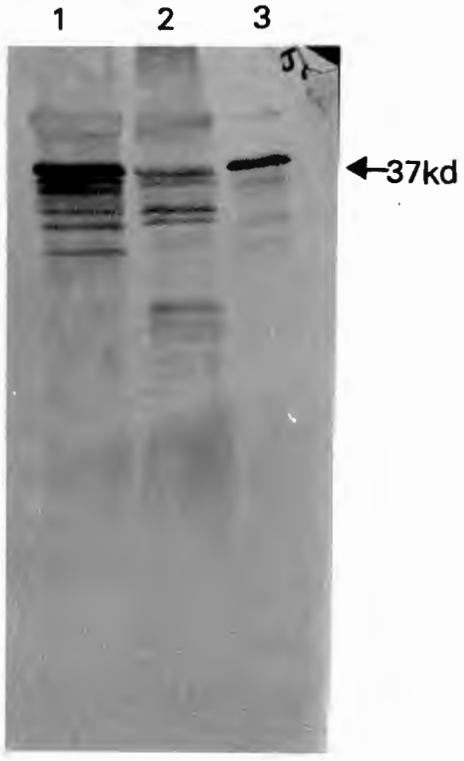
1. Antisera were diluted in antibody dilution buffer(IX.A.3.(a)) and conjugate in post-coating buffer(IX.A.3(c)).
2. 'Hentie'-isolates were extracted for CMV and diluted in post-coating buffer (IX.A.3.(c)).
3. Absorbance at 405 nm of 1/4 extract dilution of plants infected with Hentie isolate. Positive reactions have $OD_{405nm} > 0,2$ which is double the reading compared to uninfected maize.
4. The antisera, anti-CMV-Y and anti-CMV-Q, were early bleedings which could explain the negative results.

The 'Hentie' maize isolate contained antigens which reacted positively in the DAS-ELISA with antisera against several strains of CMV. Positive reactions were recorded for anti-CMV-Is, -S, -K and -Y. The strongest reaction was that of anti-CMV-K with 'Hentie'-isolate propagated in maize.

(4) Further evidence to confirm the identity of the two viruses was obtained using IEB (Figure III. 8.a and b). Anti-MDMV-B-ST serum strongly recognized the 37 kd MDMV-protein and its accompanying polypeptides in a 'Hentie' preparation. This protein corresponds to that of the MDMV-B-ST standard (Figure III. 8.a, lane 1). Anti-MDMV-B serum produced a similar result (Figure III. 5.b). Anti-'Hentie' serum recognised the 24,5 kd CMV protein in the 'Hentie'-isolate propagated on to N. clevelandii and squash (Figure III. 8.b, lanes 3 and 4). In addition, antibodies in the anti-'Hentie' serum recognised the CMV and MDMV proteins in standard purified preparations of these viruses (lanes 1 and 5).

D. CONCLUSION:

Analysis of three randomly collected field infected maize plants showed that the plants were infected by two distinct viruses i.e. maize dwarf mosaic virus and cucumber mosaic virus. The samples were collected from three geographically distant



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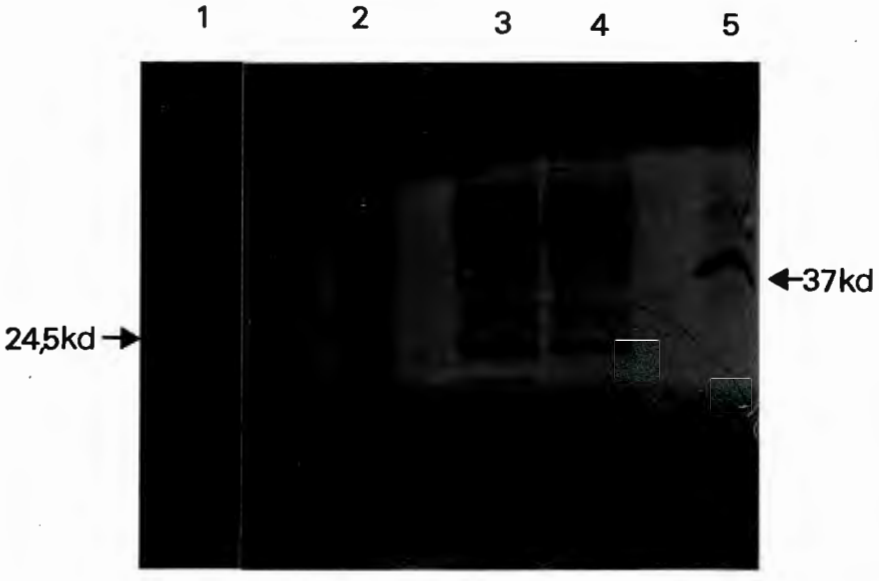


b

Figure III. 8 a and b: Immunoelectroblot assay of 'Hentie'-isolate.

(a) Blot probed with anti-MDMV-B-ST serum at a 1/30 dilution

Lane 1 - 'Hentie'-maize

2 - 'Roodeplaat B' maize

3 - MDMV-B-ST standard

Note strong recognition of 37 kd protein in lanes 1 & 2.

(b) Blot probed with 'Hentie' serum (IX.D.4) used at a 1/30 dilution. Recognition of the CMV and MDMV proteins is evident (lanes 1 and 5 respectively).

Lane 1 - CMV-Y standard

2 - CMV-K standard

3 - Hentie-isolate propagated on N. clevelandii

4 - Hentie-isolate propagated on squash

5 - MDMV-B-ST standard

Empty lanes are unmarked.

regions. It is of interest that in the Potchefstroom district, crops such as tobacco, cucurbits and legumes are grown, all of which are good hosts for CMV. This implies that the maize CMV could have originated in these alternate crops. The 'Roodeplaat' infected maize originated from the Horticultural Research Institute where many crops grow side by side in an urban area, and in this instance the maize was surrounded by numerous plants known to be CMV-hosts. Little information was available on the environment where the 'McArthur'-isolate was collected. Therefore no explanation can be given as to the origin of this CMV infecting maize.

Various factors must be considered to account for the doubly infected maize.

(1) Seed infection; there have been reports of both MDMV and CMV seed transmission (Knox, 1983; Chauhan, 1985). Primary infection from seedborne virus could lead to secondary spread if aphids colonized such plants.

(2) Aphid transmission; both MDMV and CMV can be non-persistently transmitted by several aphid species (R. maidis, R. padi, M. persicae, Matthews, 1981). These aphids commonly occur in maize fields (von Wechmar, pers. comm.) See also Chapter V.F.

(3) In some agricultural regions other crops, which are also hosts for CMV, are grown near maize fields. Early CMV-infected cucurbit or tobacco crops may harbour populations of different aphids which could migrate to the maize fields. Although certain aphid specificities have been recorded for some viruses, CMV transmission is less aphid species-specific.(See Chapter V.F.).

In all three maize isolates it was possible to maintain the double infection under laboratory conditions. Several questions arose as to the interaction of the two viruses. An investigation was therefore initiated at a cellular level in an attempt to study a possible interaction of MDMV and CMV. (See Chapter VI.E)

CHAPTER IV

CUCUMBER MOSAIC VIRUS AND MAIZE DWARF MOSAIC IN MAIZE.

A. INTRODUCTION

In 1934, Wellman described a corn disease in USA and attributed it to southern celery mosaic virus. Price (1935) showed it to be a strain of cucumber mosaic virus. Stoner (1949) investigated aphid transmission of CMV infected corn in California. After these early reports, little has been recorded on cucumber mosaic virus in maize and no records could be found relating CMV infection to severe disease or yield loss. This is not surprising as symptoms of CMV infection in maize are insidious (von Wechmar, unpublished) and one can easily understand that infections with this virus were not considered to be important. In many instances dwarfing is the only visible sign of the presence of CMV in mature plants. In contrast, early seedling infection causes bleached areas or necrotic spots in sap-inoculated plants. Aphid transmission of CMV to maize may produce symptoms different to those which result after sap transmission (von Wechmar and Knox, 1984, unpublished results).

In the 1983/84/85 seasons it was repeatedly found that CMV and MDMV could co-exist in natural field infections (Chapter III). This finding led to the decision that a more detailed investigation of the two viruses, in single and in mixed infections of maize, could be of interest.

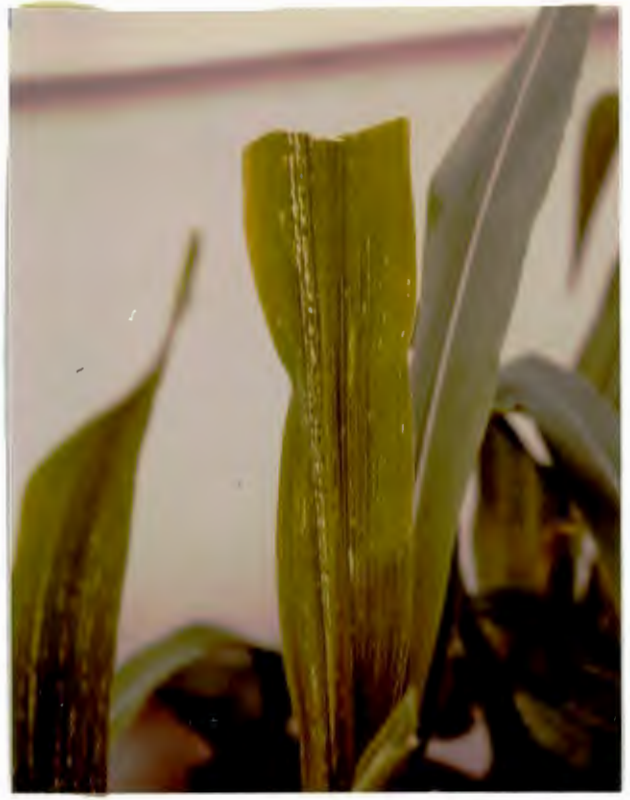
At the time of this study a pure CMV-isolate from field collected plants (doubly infected with CMV and MDMV) was not available, since no attempt had been made at that time to separate the two viruses. For this reason different strains of CMV from the departmental collection were used to sap-inoculate different maize cultivars to investigate which strain infected maize most readily. It was hoped that such a strain could then be used in protoplast studies. To ensure that the most suitable strain was selected, comparisons were made by inoculating CMV-Y, CMV-K and CMV-Lupin-K5, on to a selected host range i.e. squash, glutinosa tobacco and maize. Table IV.1 summarizes the symptoms produced by the CMV strains. Figures IV.1.a and b show the severe distortion which CMV-K causes when sap-inoculated on to glutinosa tobacco and squash. This strain of CMV had previously been reported to infect maize (Rao and Francki, 1982; Tien Po, 1982). In our experience CMV-K gave rise to mosaic symptoms in some maize cultivars tested (Figure IV.1.c) while in others it caused only chlorotic and necrotic lesions on inoculated leaves. Secondary leaves, although containing virus, were symptomless. It was decided that CMV-K would be the best strain to use in this study as its presence could be easily recognised by symptom expression in maize. In addition, CMV-K in glutinosa tobacco causes a severe shoe-string effect in later stages of infection which differentiates it from symptoms produced by any other CMV strain in the department (Figure IV.1.a).

TABLE IV.1: Symptoms produced by three different strains of CMV sap-inoculated to three different hosts

Strain of CMV	Host		
	Squash	glutinosa tobacco	Maize-A
CMV-Y	Yellow-mosaic, dwarfing, distortion of leaves	Bubbly 'green islands' in yellow chlorotic background	Symptomless, except dwarfed
CMV-Lupin-K5	-	Mild green mosaic dwarfing	Symptomless except dwarfed
CMV-K	Severe distortion, crumpling of leaves (Figure IV.1b)	Green mosaic, dwarfing, distortion and crinkling of inoculated leaves and severe shoe-string effect in later growth (Figure IV.1.a)	Interrupted streaks and mosaic (Figure IV.1.c)

a

b



c

Figure IV.1 a, b and c: Symptoms produced by CMV-K sap-inoculated on to three different hosts.

(a) Severe shoe-string effect produced on mature glutinosa tobacco.

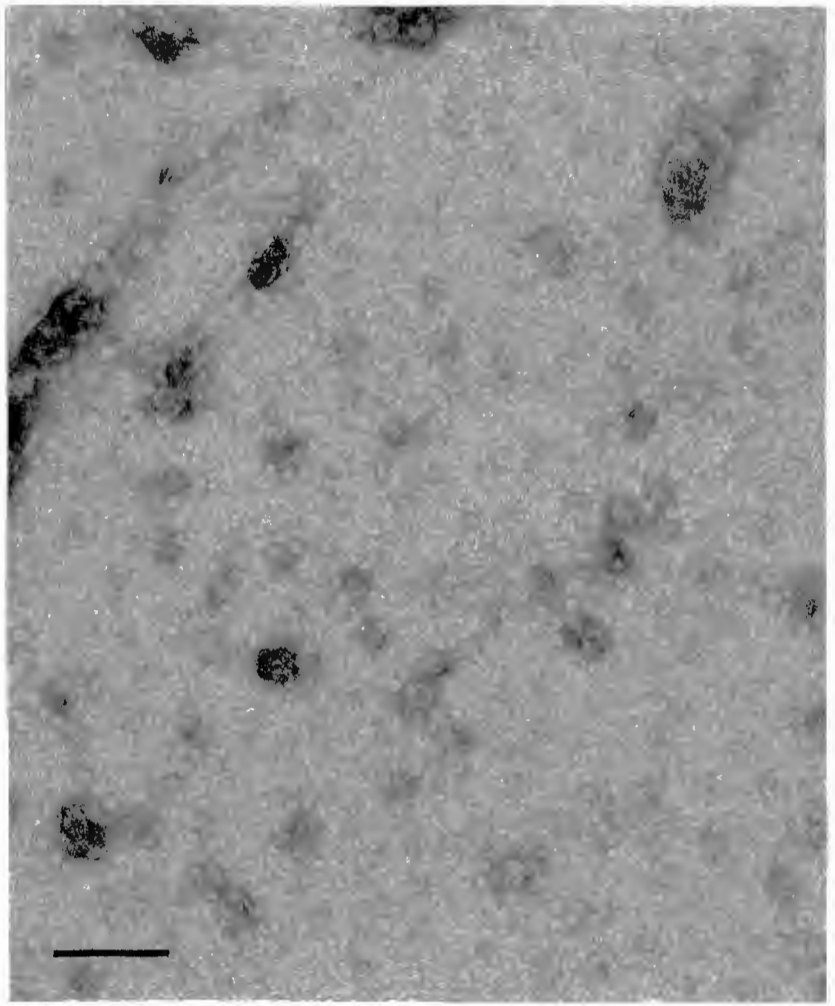
(b) Distortion and crumpling on squash.

(c) Interrupted streak mosaic on maize.

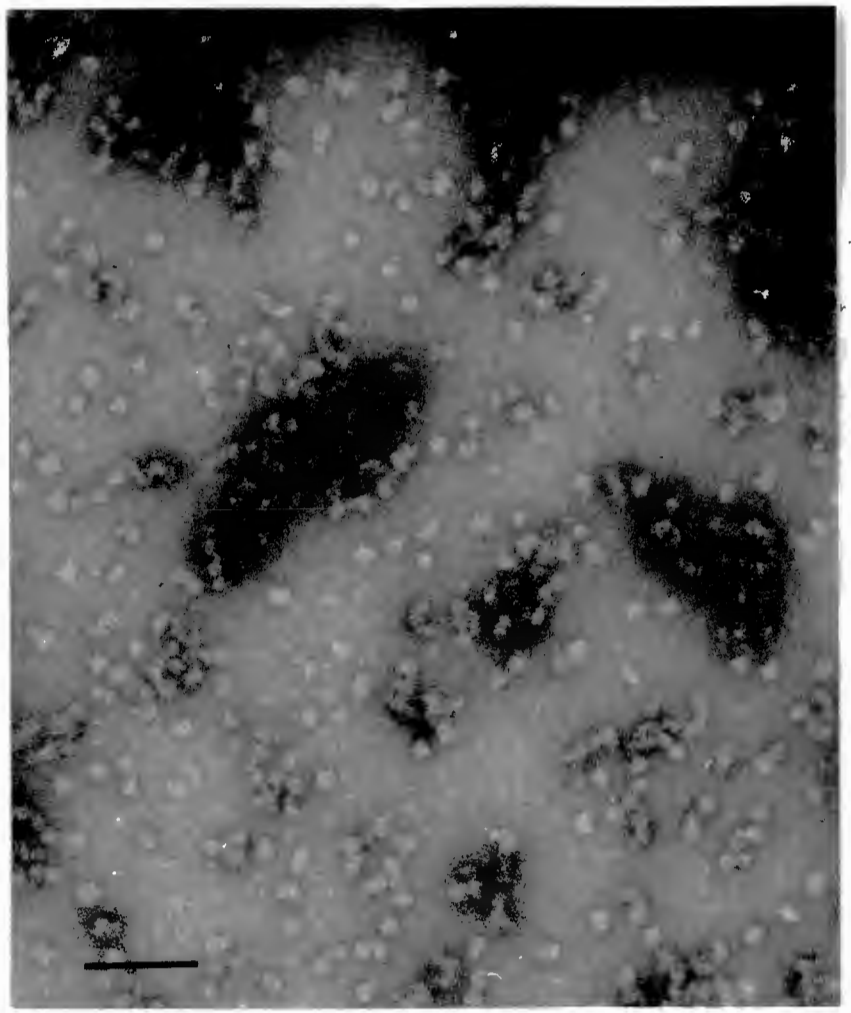
B. SOME CHARACTERISTICS OF CMV-K

Once it had been determined that CMV-K was the most suitable strain of CMV for use in this study (see Chapter IV.A and Table IV.1), a few of its properties were briefly investigated. Propagation was in glutinosa tobacco (see IX.D.1.(c)). Virus was purified routinely from squash according to the CMV method (see IX.D.3.(a)). A spectrophotometric UV scan of the purified virus is illustrated in Figure IV.2.a. Extracted virus was treated with 2% formaldehyde to stabilize the virus particles (Francki and Habili, 1972; Kaper and Waterworth, 1981). Stabilized CMV-K was used for immunization of rabbits and as standard controls in ELISA tests (Figure IV 2.b). Unless stabilized, many of the virus particles in the purified preparation degraded soon after extraction (approximately two days) (Figure IV.2.c).

The relative concentration of CMV-K when propagated in different hosts was calculated (Table IV.2). A purified preparation of known concentration was titrated and the saps of infected hosts were calibrated against this using DAS-ELISA.



a



b

Figure IV.2 a, b and c:

(a) Electron micrograph of CMV-K extracted from squash and treated with 2% formaldehyde. Magnification was 90 000. Bar = 100 nm.

(b) Electron micrograph of unstabilized CMV-K. Mainly degraded particles. Magnification was 90 000. Bar = 100 nm.

(c) An ultraviolet absorption scan of CMV-K extracted from glutinosa tobacco by the CMV method (IX.D.3.(a)). The virus preparation was diluted 1/100 in borate buffer, pH 8,0.

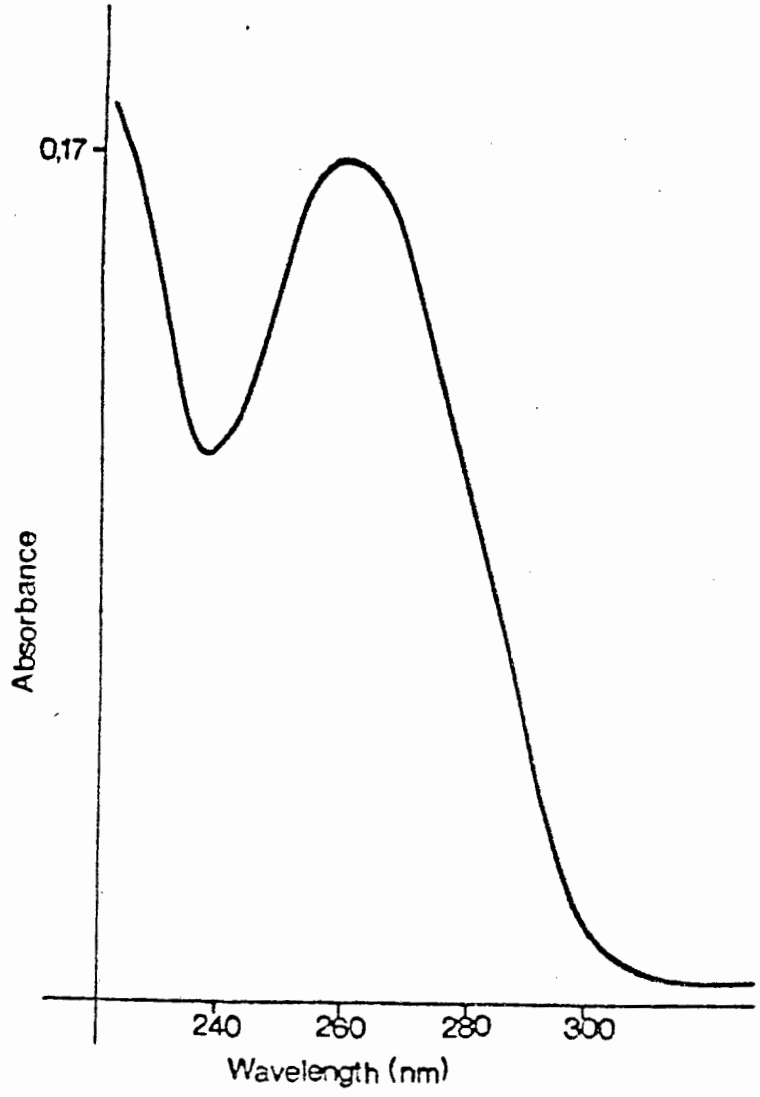


Table IV.2: Concentration of CMV-K when propagated on different hosts.

Host	Concentration of CMV-K[1] (mg/ml) sap
squash[2]	4 mg/ml
glutinosa tobacco leaves[3]	1,4 mg/ml
maize-A leaves[4]	1,5 mg/ml

1. The concentration of CMV-K in each was determined using DAS-ELISA (IX.D.5). A titration of a known concentration of purified CMV-K was carried out and the unknown amount of CMV-K in the saps of different hosts calculated.
2. CMV-K was propagated for 10 days on squash. The virus was extracted using the CMV method (IX.D.3.(a)).
3. Glutinosa tobacco was sap-inoculated with CMV-K. After 10 days the first three leaves were tested.
4. Maize-A seedlings were sap-inoculated with CMV-K. After 7 days plants were crushed and tested.

To investigate the infectivity of the virus,it was extracted and purified from infected squash plants by the CMV method (IX.D.3.(a)). This preparation was stored at 4°C in borate buffer, pH 8 (IX.A.2.(b)). At two-day intervals, for 12 days, it was inoculated on to glutinosa tobacco. Table IV.3 summarizes the results obtained.

Table IV.3: Infectivity and longevity of purified CMV-K over 12 days.

Days after isolation[1]	Number of plants showing symptoms / Number of plants inoculated
0	8/8
2	8/8
4	8/8
6	5/8
8	4/8
10	1/8
12	0/8

1. CMV-K was extracted from infected squash and the purified preparation stored at 4°C in borate buffer, pH 8.

It had been anticipated that, in a later stage of the programme, maize protoplasts would be inoculated with extracted RNA. For this reason a preliminary investigation of RNA extraction from CMV was done. The method used was an adaption from that of Brisco et al., (1985). (Miss C. Williamson modified this method for the extraction of RNA from the aphid virus, Rhopalosiphum padi virus (RhPV), unpublished). The method is described in detail in IX.D.9.

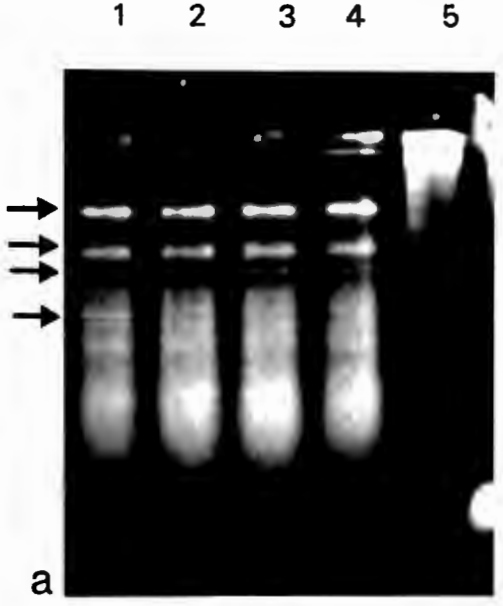
Figure IV.3.a shows the RNA species which were isolated from CMV-K which had been propagated on and purified from squash. Accurate molecular weight determinations could not be calculated by agarose gel electrophoresis; denaturing gel electrophoresis is required for this purpose.

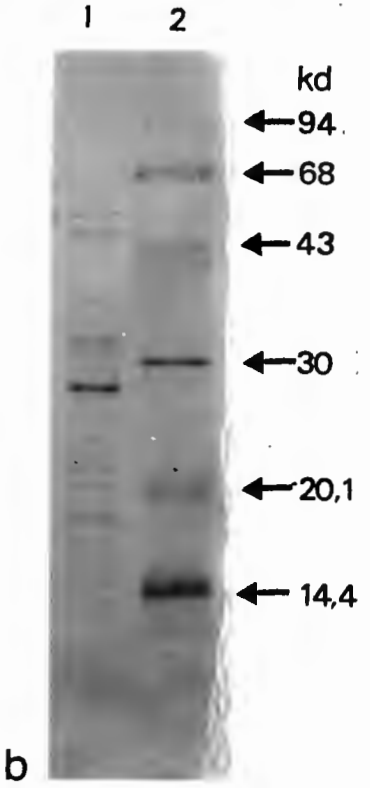
To determine the protein profile of CMV-K, polyacrylamide gel electrophoresis of a purified preparation of CMV-K was done. Figure IV.3.b illustrates the 24,5 kd protein.

It was hoped that CMV-K could be introduced into protoplasts via liposomes. In order to detect encapsulation of the virus in the liposomes, a preliminary investigation of radiolabelling of the virus was done. The radiolabel used was ^{35}S protein labelling reagent (^{35}SLR, Amersham) and the method for its incorporation is described in IX.D.10. Figure IV.3.c illustrates an autoradiograph of the labelled CMV-K.

C. SUITABILITY OF MAIZE HOSTS TO SUPPORT CMV REPLICATION

The yield of virus which could be isolated from two different maize hybrids was calculated to ascertain which of the two was a better host for CMV. Different strains of CMV (CMV-Y, CMV-K and CMV-Lupin-K5) were sap-inoculated from glutinosa tobacco to 7 day old seedlings of two maize hybrids. After 10 days the maize plants were harvested and the virus extracted by the CMV method


1
2
3
4
5
a


1
2
kd
94
68
43
30
20,1
14,4
b

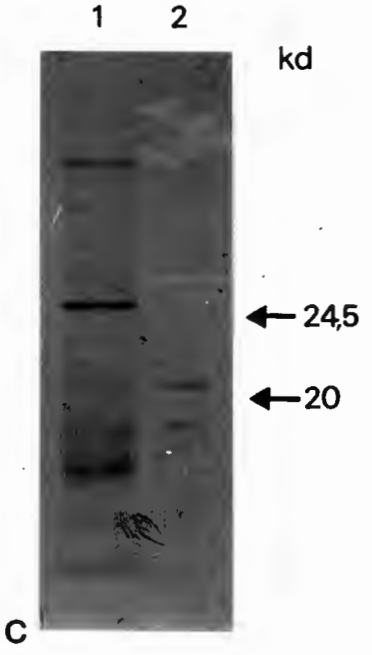

1
2
kd
24,5
20
c

Figure IV.3 a, b and c: Electrophoresis of CMV-K nucleic acid and protein.

(a) CMV-K RNA electrophoresed on a 1,5% agarose gel. The gel was run for 2 hours at 50 volts and stained with ethidium bromide.(IX.D.9)

Lane 1 - CMV-K RNA (2ul)

2 - CMV-K RNA (3ul)

3 - CMV-K RNA (6ul)

4 - CMV-K RNA (8ul)

5 - M_r marker[1]

(b) PAGE-gel electrophoresis of a CMV-K preparation extracted from squash by the CMV method. Gel was stained with Coomassie blue (IX.D.6).

Lane 1 - CMV-K

Lane 2 - Protein M_r, marker (Pharmacia) Phosphorylase B 94 kd; bovine serum albumin 68 kd; ovalbumin 43 kd; carbonic anhydrase 30 kd; soybean trypsin inhibitor 20,1 kd; lactalbumin 14,4 kd.

The 24,5 kd protein of CMV-K is evident.

(c) Autoradiograph of CMV-K radiolabelled using ^{35}SLR (IX.D.10).

Lane 1 - CMV-K

2 - BMV-ST

1. Note: Accurate molecular weight determinations were not possible to calculate as M_r marker did not run on gel (Electran, BDH Chemicals).

(IX.D.3.(a)). The yield of each virus strain was calculated by extrapolation of UV absorbance scan readings ($E^{0.1\%}_{260\ nm} = 5$; Francki et al., 1979) and the fresh leaf weights. Table IV.4 summarizes the results obtained.

Table IV.4: Yields of three different strains of CMV (-Y; -K and -Lupin-K5) extracted from two maize hybrids.

	Virus yield (mg/kg leaf tissue)	
Strain of CMV	Maize-A	Maize-B
CMV-Y	109	90
CMV-K	60	40
CMV-Lupin-K5	20	10

To confirm the relative amounts of the different CMV strains in the two maize hosts, DAS-ELISA was carried out. Virus was extracted from 14 day old maize plants by the CMV method (IX.D.3(a)) and a two-fold serial dilution of each virus extract prepared. The dilutions were reacted with antisera, homologous to each strain of CMV. Figure IV.4 a, b and c illustrates the results obtained. To summarize it appears that CMV-K replicates

to a greater extent in maize-A than in maize-B (Figure IV.4.a). Maize-A is a better host for CMV-Y than maize-B (Figure IV.4.b). In comparison to CMV-K and CMV-Y, it is evident that CMV-Lupin-K5 infects maize-A and maize-B less efficiently.

Purified extracts of the two maize hybrids infected with different CMV strains were subjected to PAGE gel electrophoresis(IX.D.6) and enzyme immuno-electroblotting (IX.D.7). The results can be summarized as follows;

(a) CMV-Y could be detected in both maize hybrids (Figures IV.5.a and b)

(b) CMV-K was evident in both maize hybrids by Coomassie staining of acrylamide gels (Figures IV.6.a). When equal quantities of the maize extracts were immunoelectroblotted usng anti-CMV-K serum, more antigen could be detected in maize-A than in maize-B (Figure IV.6 b).

(c) CMV-Lupin-K5 could not be detected in either of the maize extracts by Coomassie staining (Figure IV.7). Probing an immunoelectroblot with homologous anti-CMV serum failed to recognise CMV protein bands in either of the extracts.

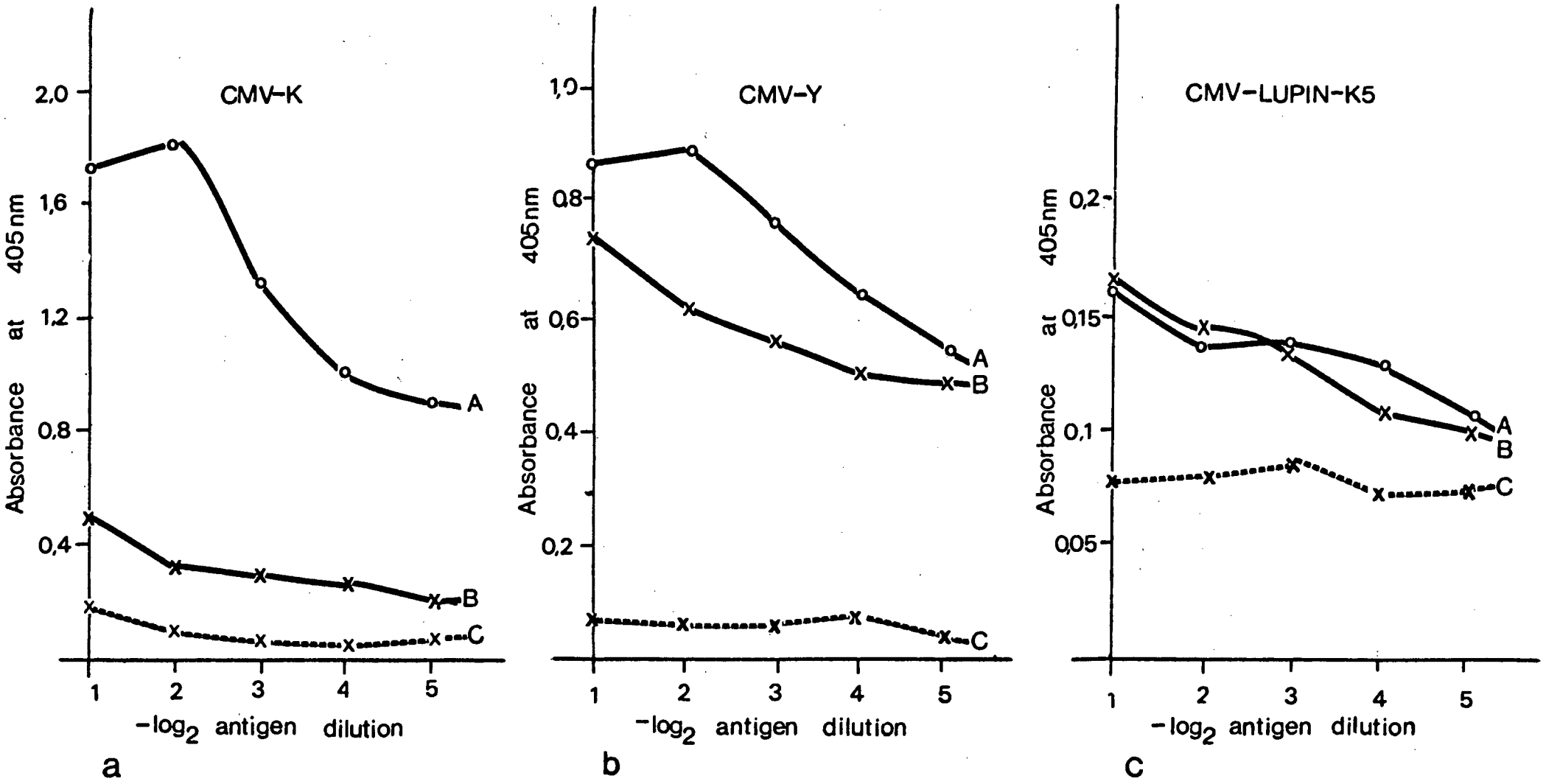
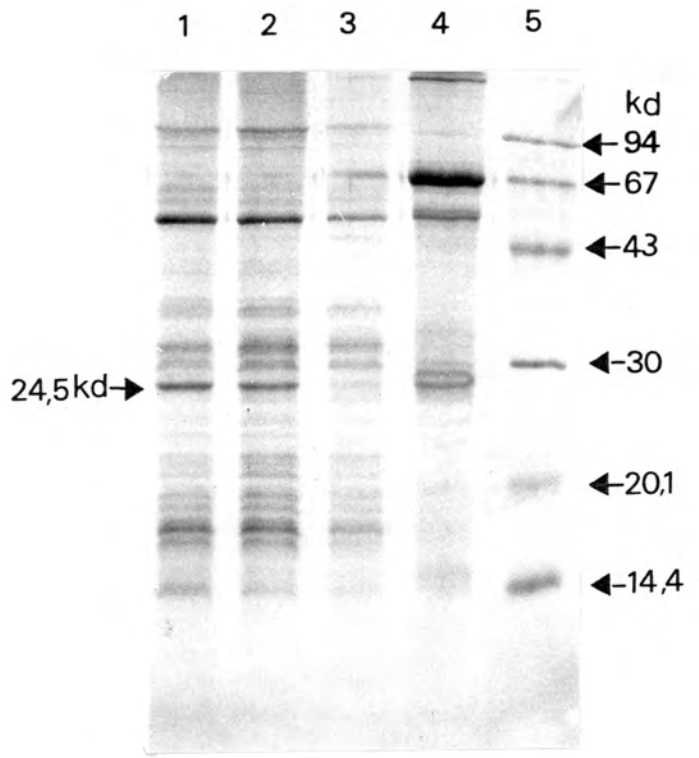


Figure IV.4 a, b and c: Reactions of different strains of CMV (-K, -Y and Lupin-K5) in two different maize hybrids (A and B) with homologous antisera in DAS-ELISA. Uninfected Maize-A was used as a control (C). The IgG and conjugate controls were as follows; anti-CMV-K 1/300; anti-CMV-Y 1/300; anti-CMV-Lupin-K5 1/400.



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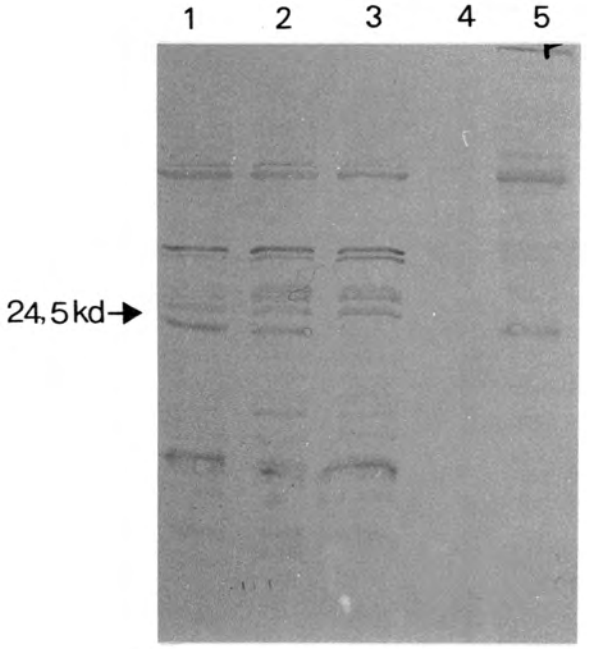


b

Figure IV.5 a and b: CMV-Y on maize.

a) Coomassie stained gel of extracts form maize-A and maize-B sap inoculated with CMV-Y.

Lane 1 - Extract from maize-A

2 - Extract from maize-B

3 - Uninfected maize (maize-A) control

4 - CMV-Y on glutinosa tobacco (leaf crush)

5 - Protein M_r marker (Pharmacia) Phosphorylase B 94 kd; bovine serum albumin 67 kd; ovalbumin 43 kd; carbonic anhydrase 30 kd; soybean trypsin inhibitor 20,1 kd; lactalbumin 14,4 kd.

b) Immuno-electroblot of CMV-Y infected maize. Anti-CMV-Y antisera was used at a 1/30 dilution.

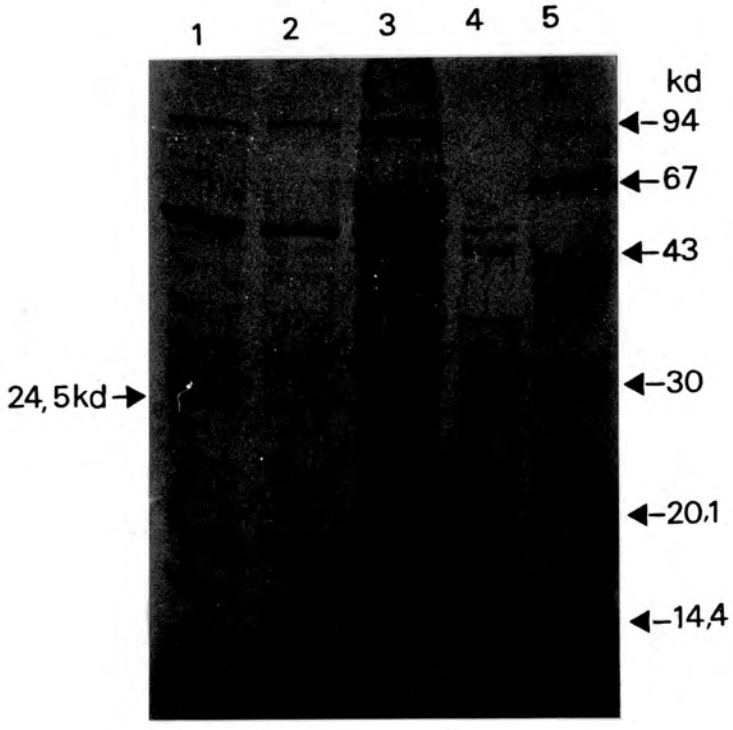
Lane 1- Extract from maize-A

2- Extract from maize-B

3- Uninfected maize (maize-A)

4- Uninfected maize (maize-B)

5- CMV-Y on glutinosa tobacco (leaf crush) control



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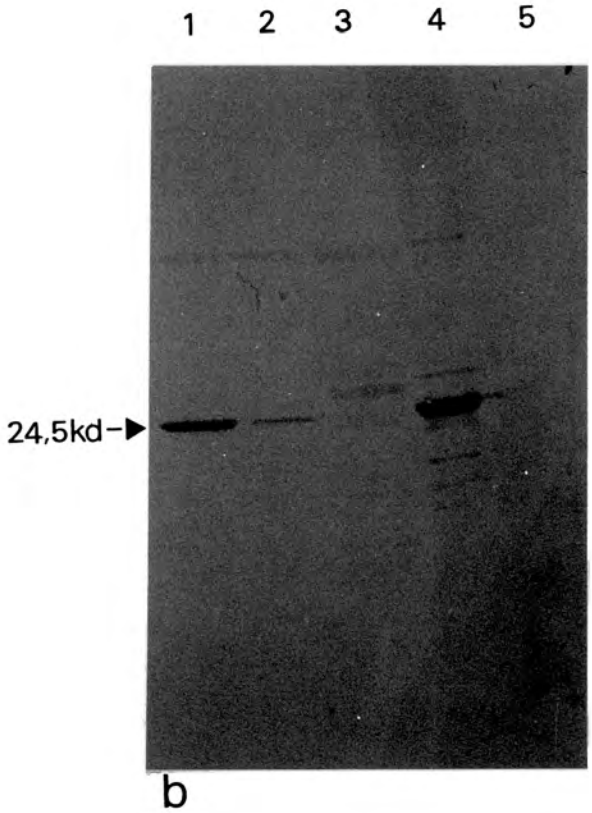


b

Figure IV.6 a and b: CMV-K on maize.

a) Coomassie stained gel of extracts of CMV-K infected maize.

Lane 1 - Extract from maize-A

2 - Uninfected maize (maize-B) control

3 - Extract from maize-B

4 - CMV-K purified from glutinosa tobacco

5 - Protein M_r marker (Pharmacia) Phosphorylase B 94 kd; bovine serum albumin 67 kd; ovalbumin 43 kd; carbonic anhydrase 30 kd; soybean trypsin inhibitor 20,1 kd; lactalbumin 14,4 kd.

b) Immuno-electroblot of CMV-K infected maize. Anti-CMV-K antisera was used at a 1/30 dilution.

Lane 1 - Extract from maize-A

2 - Extract from maize-B

3 - Extract from uninfected maize (maize-B)

4 - CMV-K purified from glutinosa tobacco

5 - Extract from uninfected maize (maize-A)

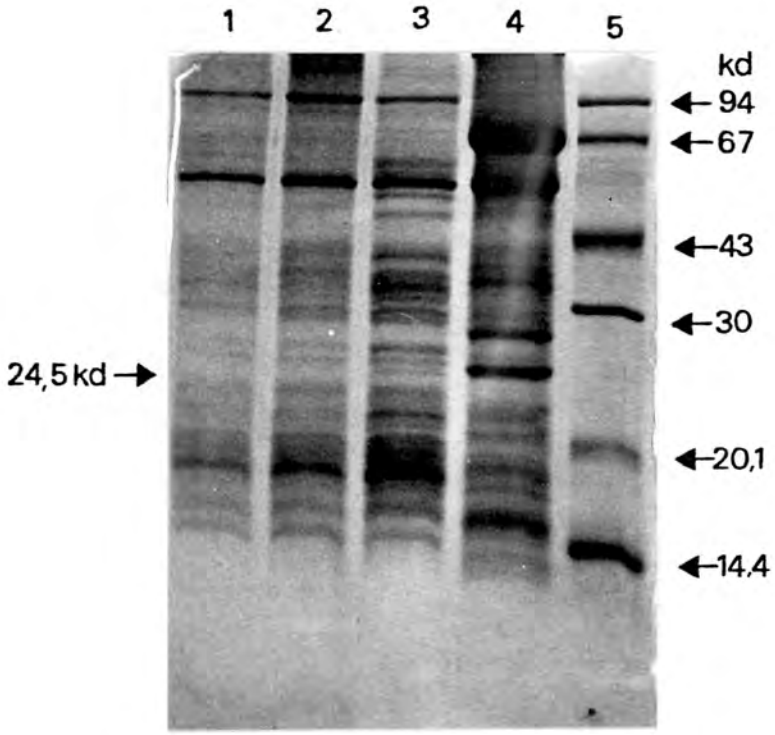


Figure IV.7: Coomassie stained gel of extracts from CMV-Lupin-K5 infected maize.

Lane 1 - Extract from maize-A

2 - Extract from maize-B

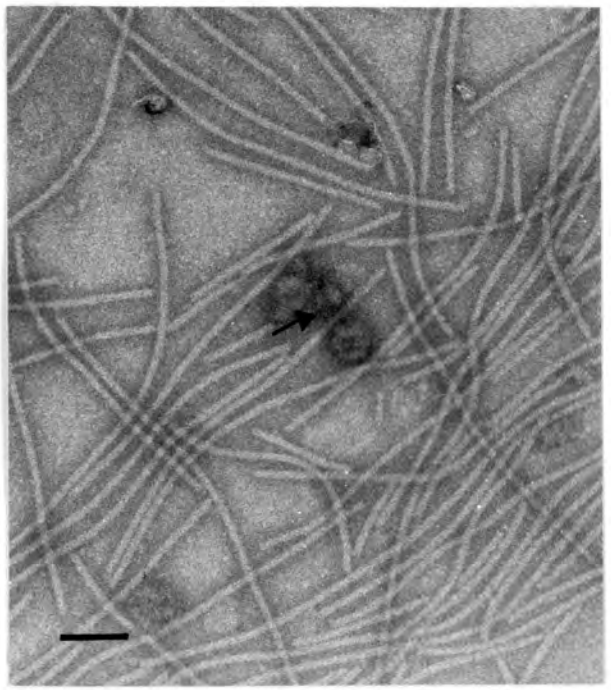
3 - Uninfected maize (maize-A) control

4 - Protein M_r marker; phosphorylase B 94 kd; bovine serum albumin 67 kd; ovalbumin 43 kd; carbonic anhydrase 30 kd; soybean trypsin inhibitor 20,1 kd; lactalbumin 14,4 kd.

D. SOME CHARACTERISTICS OF MDMV-B-ST

Characterization of MDMV isolates and strains had previously been determined in the Department (Chauhan, 1985). As the data was readily available the work was not repeated for this project. The isolate used was MDMV-B-ST. Figure IV.8.(a), (b) and (c) illustrate some of its characteristics. Figure IV.8.(a) is an electron micrograph showing the filamentous particle; (b) is a typical UV scan of a purified MDMV-B-ST preparation and (c) is an RNA gel.

Natural double infections of MDMV-B and CMV have already been discussed in Chapter III. The interaction of the two viruses in maize protoplasts is reported in Chapter VI.E.



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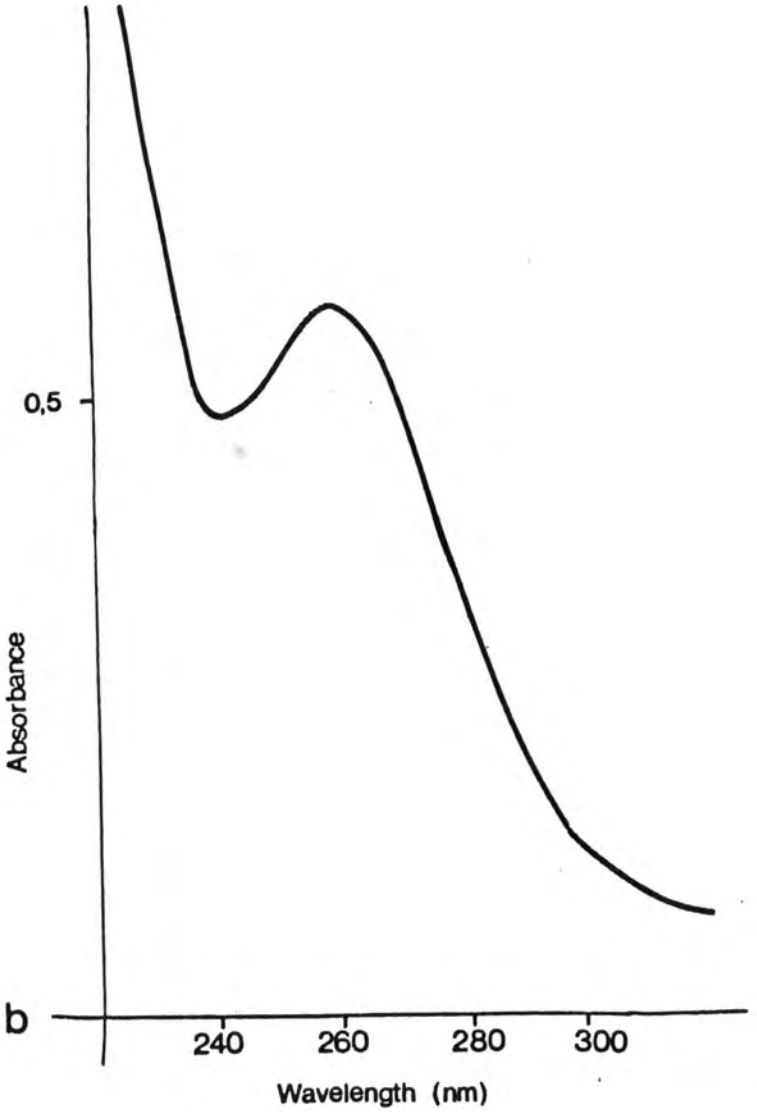
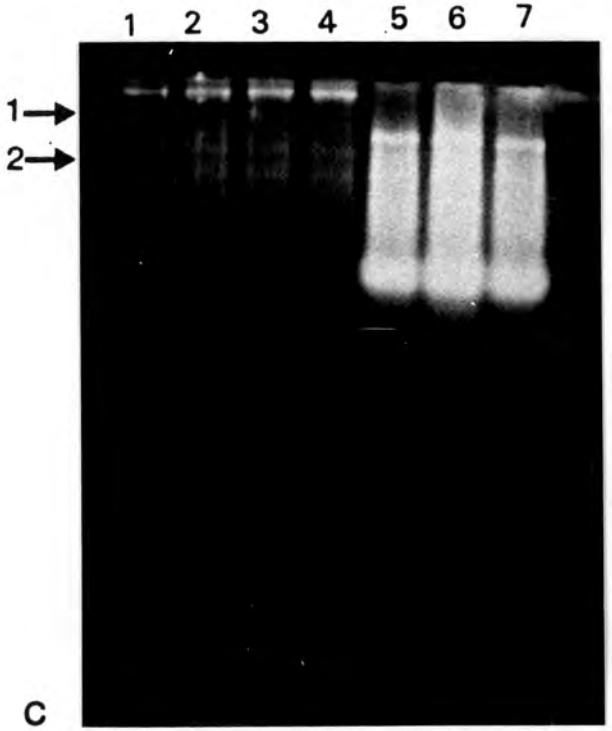


b



c

Figure IV.8:

a) Electron micrograph of MDMV-B-ST purified from cv.KEP by the method described in IX.D.3(b). The sample was stained with uranyl acetate and viewed at a magnification of 90 000x. Bar = 100 nm.

b) An ultraviolet scan of purified MDMV-B-ST.

c) MDMV-B-ST RNA electrophoresed on a 1,5% agarose gel. The gel was run for 2,5 hours at 50 volts and stained with ethidium bromide.

Lane 1 - MDMV-B-ST RNA (4 ul)

2 - MDMV-B-ST RNA (6 ul)

3 - MDMV-B-ST RNA (10 ul)

4 - MDMV-B-ST RNA (12 ul)

5 - M_r marker (Electran BDH Chemicals). Only 1,75 x 10^6 da marker (r-RNA, 28S) visible.

6 - M_r marker

7 - M_r marker[1]

1. Accurate molecular weight of MDMV-B-ST RNA could not be calculated.

1 = Position of MDMV-RNA

2 = Position of 28 S M_r marker

Note presence of two lower bands in lanes 1 to 4. Possibly contamination with a second virus (see Chapter VII, Discussion).

CHAPTER V

BIOLOGICAL ASPECTS OF DOUBLE INFECTION OF MAIZE WITH MAIZE DWARF MOSAIC VIRUS STRAIN-B-ST AND CUCUMBER MOSAIC VIRUS STRAIN-K

A. INTRODUCTION

In Chapter III the natural double infection of maize with MDMV and CMV was reported. Although only three incidences were investigated, this phenomenon was observed frequently in natural infections (von Wechmar, Maize Virus Progress Report, 1984-85, 1985-86). It is uncertain whether seedborne viruses play a role in this phenomenon and whether a certain inter-relationship is involved in these double infections. To gain a better understanding of the role of each virus in single and double infections, and the interaction between the two in the latter, laboratory tests were carried out. This chapter reports on the biological aspects that were studied. Maize seedlings were sap-inoculated i) singly with either CMV or MDMV, ii) with a 1:1 mixture of the viruses or iii) with one virus followed after a time interval by the other. The resulting infected plants were tested for the presence of each virus by DAS-ELISA. By recording the number of doubly infected plants it was hoped that synergism or cross protection between the two viruses might be observed.

B. SAP-INOCULATIONS

In all cases for sap-inoculation, CMV-infected glutinosa tobacco leaves were ground up in CMV buffer (IX.A.2(d)) and MDMV-B-ST-infected maize leaves (cv.KEP), in 0,1 M phosphate buffer (pH 7) (IX.A.1.(a)). Maize seedlings were inoculated at the 2-3 leaf stage and squash plants at the dicotyledonous stage. The plants were kept at 25°C in plant growth rooms (14 hour/10 hour day/night cycle) until tested for the presence of virus by DAS-ELISA (IX.D.5).

1. Inoculums used

The following inoculums were used to inoculate young seedlings of maize-A and -B.

a) CMV-K propagated on glutinosa tobacco

b) MDMV-B-ST propagated on maize cv. KEP.

c) CMV-K and MDMV-B-ST simultaneously by mixing the two sap inoculums (a) and (b) 1:1 (volume:volume).

2. Sequence of application

The above inoculums were applied in the following sequences.

a) CMV-K primary infection followed by MDMV-B-ST as secondary infection at 4, 8, 10 and 12 day intervals post-CMV infection.

b) MDMV-B-ST primary infection, CMV-K as secondary infection at 4, 5, 6 and 7 days post MDMV-B-ST infection.

3. Inoculations with sap obtained from known established double infections.

Plants that had been shown by DAS-ELISA to be doubly infected when sap-inoculated with a mixture of MDMV and CMV saps (see V.B.1.c) were targeted for this experiment. It was of interest to ascertain whether a double infection could be maintained in maize. Sap was prepared from doubly infected maize seedlings and inoculated on to a selection of maize hybrids i.e. maize-A, -B and KEP.

Regrettably squash was omitted in this experiment. In retrospect it could have been interesting to observe whether CMV would have caused a severe infection in this host after being cycled over maize.

C. DAS-ELISA

Sap-inoculated maize plants were squashed using a roller press (G.Pollahne, Germany). Individual plants were crushed between two rollers and the sap collected in Wasserman tubes. The saps were kept on ice until used. DAS-ELISA (IX.D.5) was set up to test for the viruses in the saps. Anti-MDMV-B-ST and anti-CMV-K IgG and corresponding conjugates were used in all the tests.

D. RESULTS

1. Inoculums

Table V.1 presents the results obtained when maize -A and -B were singly or doubly infected with CMV-K and MDMV-B-ST (V.B.1). The plants were tested by DAS-ELISA 10, 11, 12 and 13 days after inoculation. It is evident that a high percentage of the plants could be infected with CMV-K or MDMV-B-ST. When both viruses were present simultaneously in the inoculum, there did not appear to be a synergistic effect between the two; nor was cross-protection observed.

The fact that neither CMV nor MDMV were recognised as seed-transmitted in Table V.1 does not exclude the possibility that latent infections could be seedborne. Chauhan (1985) showed that seedborne virus was more commonly detected in slow germinating/growing maize compared to fast germinating/growing maize seedlings. For experiments in this section, only fast germinating seedlings were selected to avoid seedlings which contained seedborne virus and would confuse results if present.

2. Sequence of inoculation

Table V.2 presents the results when maize-A and maize-B were sap-inoculated with MDMV-B-ST and CMV-K in different sequences (V.B.2) and the effect on infection of different time intervals between inoculations.

Table V.1 a and b: Detection by DAS-ELISA of MDMV-B-ST and CMV-K in single and double infections of two different maize hybrids.

a) Maize-A[1]

Inoculum	Interval post-inoculation (days)	DAS-ELISA results			
		CMV-K		MDMV-B-ST	
		No. positive / No. tested	% infected	No. positive / No. tested	% infected
CMV-K only	10	10/10	100%	0/10[3]	0
"	11	10/10	100%	0/10	0
"	12	7/7	100%	0/7	0
"	13	8/8	100%	0/8	0
MDMV-B-ST	10	0/8	0[4]	8/8	100%
"	11	0/8	0	8/8	100%
"	12	0/7	0	7/7	100%
"	13	0/7	0	7/7	100%
MDMV-B-ST and	10	7/8	88%[5]	5/8	63%[5]
CMV-K	11	9/16	56%	11/16	69%
simultaneously	12	10/16	63%	12/16	75%
"	13	16/16	100%	13/16	81%

b) Maize-B[1]

Inoculum	Days post-inoculation	CMV-K		MDMV-B-ST	
		No. positive[2] / No. tested	% infected	No positive / No. tested	% infected
CMV-K only	10	7/7	100%	0/7	0[3]
"	11	8/8	100%	0/8	0
"	12	8/8	100%	0/8	0
"	13	8/8	100%	0/8	0
MDMV-B-ST	10	0/8	0[4]	8/8	100%
only	11	0/8	0	8/8	100%
"	12	0/8	0	6/8	75%
"	13	0/7	0	5/8	71%
MDMV-B-ST and	10	6/8[6]	75%	4/8[6]	63%
CMV-K	11	11/16	69%	10/16	63%
simultaneously	12	5/16	31%	11/16	69%
"	13	10/14	71%	9/14	64%

1. See IX.C.
2. The table only presents the results as positive or negative for CMV-K. The actual quantity of CMV in maize could be related to 1,5 mg/ml when compared to a similar inoculation of squash which gave 4 mg/ml (see Chapter IV).

3/4. Tests were conducted for MDMV-B-ST and CMV-K respectively to detect the possibility of seed-borne virus.

5. See Chapter VIII for discussion
6. Relative concentration of CMV-K and MDMV-B-ST in doubly infected plants was 1,5 mg/ml and 2,8 mg/ml respectively.

Table V.2 a and b: Detection by DAS-ELISA of MDMV-B-ST and CMV-K in single and double infections in two different maize hybrids. The second inoculation was applied at different time intervals.

a) Maize-A[1]

Inoculum	Time interval days	CMV-K		MDMV-B-ST	
		No. positive / No. tested	% infected	No. positive / No. tested	% infected
1. CMV-K only	0	29/30	96%	0/30	0
2. MDMV-B-ST	0	0/23	0[4]	23/23	100%
3. MDMV-B-ST and CMV-K simultaneously	0	32/53	60%	28/53	53%
4. MDMV-B-ST first	4 days later CMV-K	30/31	97%	30/31	97%
"	5 days later CMV-K	23/30	77%	28/30	93%
"	6 days later CMV-K	30/30	100%	23/30	78%
		28/30	94%	27/30	90%
5. CMV-K first	4 days later MDMV-B-ST	30/30	100%	10/30	33%[5]
"	5 days later MDMV-B-ST	30/30	100%	6/30	20%
"	10 days later MDMV-B-ST	21/25	84%	12/25	48%
"	14 days later MDMV-BST	19/25	76%	3/25	12%

b) Maize-B[1]

Inoculum	Time interval days	CMV-K		MDMV-B-ST	
		No. positive / No. tested	% infected	No. positive / No. tested	% infected
1. CMV-K only	0	28/28	100%	0/28	0
2. MDMV-B-ST only	0	0/30	0	30/30	100%
3. MDMV-B-ST and CMV-K simultaneously	0	32/54	59%	33/54	61%
4. MDMV-B-ST first	4 days later CMV-K	27/30	90%	23/30	76%
"	5 days later CMV-K	27/28	96%	24/28	86%
"	6 days later CMV-K	28/28	100%	22/28	79%
"	7 days later CMV-K	26/27	96%	26/27	96%
5. CMV-K first	4 days later MDMV-B-ST	17/25	68%	9/25	36%[5]
"	8 days later MDMV-B-ST	20/20	100%	8/20	40%
"	10 days later MDMV-B-ST	19/20	76%	10/25	42%
"	14 days later MDMV-B-ST	23/30	77%	5/30	17%

1. See Chapter IX.C.
2. The table presents the result as positive or negative for CMV-K. The actual quantitiy CMV-K could be related to 1,5 mg/ml in maize when compared to a similar inoculate of squash which gave 4 mg/ml (see Chapter IV). Therefore maize is a less efficient host fr CMV-K replication.

3/4. Tests were conducted for MDMV-B-ST and CMV-K to detect the possibility of seedborne virus.

5. See text and Discussion (Chapter VII).

Table V.3: Summary of Tables V.1 and 2. Results for maize-A and maize-B were combined.

	No. +ve MDMV/+ve CMV / No. tested	No. +ve MDMV/-ve CMV / No. tested	No. +ve CMV/-ve MDMV / No. tested	No. -ve CMV/-ve MDMV / No. tested
A. Maize inoculated with MDMV/CMV simultaneously				
10 days	9/16	0/16	4/16	3/16
11 days	14/37	11/37*	8/37	4/5
12 days	6/39	24/39*	4/39	5/39
13 days	16/28	4/28	8/28	0/28
B. MDMV-B-ST inoculated first; CMV-K after time interval				
4 days	49/57	1/57	6/57	1/57
5 days	50/60	3/60	6/60	1/60
6 days	44/54	0/54	10/54	0/54
7 days	51/57	3/57	2/57	1/57
C. CMV-K inoculated first; MDMV-B-ST after time interval				
4 days	11/50	6/50	30/50	3/50
8 days	10/30	0/30	17/30	3/30
10 days	22/46	0/46	14/46	10/46
12 days	5/31	3/31	16/31	7/31

* This exceptionally high result could be due to the fact that at 12 days virus replication had reached its maximum.

It is evident that when MDMV-B-ST has already established itself within the maize host, CMV-K is still able to infect a high percentage of MDMV-infected seedlings even 7 days after the primary infection. In contrast, MDMV infection appears to be impeded by the presence of CMV in both maize-A and maize-B.

3. Inoculations with sap obtained from known established double infections

Maize-A seedlings doubly inoculated with MDMV-B-ST and CMV-K were ground up in phoshate buffer (pH 7,0) (IX.A.1.(a)) and sap-inoculated on to a selection of maize types (V.B.3). The results of the sap-inoculations are presented in Table V.4.

The fact that neither CMV nor MDMV were not recognised as seed-transmitted does not exclude the possibility that latent infections could be seedborne. Chauhan (1985) showed that seedborne virus was more commonly detected in slow germinating/growing maize compared to fast germinating/growing maize seedlings. For experiments in this section, only fast germinating seedlings were selected to avoid seedlings which contained seedborne virus and would confuse results if present.

Table V.4: Sap-inoculation of doubly infected maize[1] to different maize hosts.

Host inoculated	Symptoms	CMV-K		MDMV-B-ST	
		No. infected		No. infected	
		No. tested	% infected	No. tested	% infected
Maize cv.KEP	Severe mosaic 2 plants dead	5/16	31%	16/16	100%
Maize-A	Interrupted stripes, mosaic, dwarfed	11/16	69%	16/16	100%
Maize-B	Mosaic, dwarfed	8/16	50%	16/16	100%
Control[4]	None	0/10	0%	0/10	0%

1. These plants were tested prior to use and shown to be infected with both MDMV-B-ST and CMV-K (Table V.1).
2. Infected plants were individually crushed in post-coating buffer (IX.A.3(c)).
3. The sap extracts were tested by DAS-ELISA using homologous antisera.
4. Control = uninfected maize-A.

4. Electron microscopy

A leaf-dip preparation of a maize seedling, shown by DAS-ELISA to contain both MDMV and CMV, was examined by electron microscopy. A mixture of anti-MDMV-B-ST and anti-CMV-K serum was used to trap the virus particles. Both filamentous and spherical particles were detected (see Figure V.1).

It is of interest that when the preparation was "decorated" with anti-MDMV-B-ST serum the CMV particles were highlighted adhering to the filamentous particle. At that time experience with the decorating technique was lacking (IX.D.8.(c)). The fact that anti-MDMV serum recognized CMV particles is discussed in Chapter VIII.

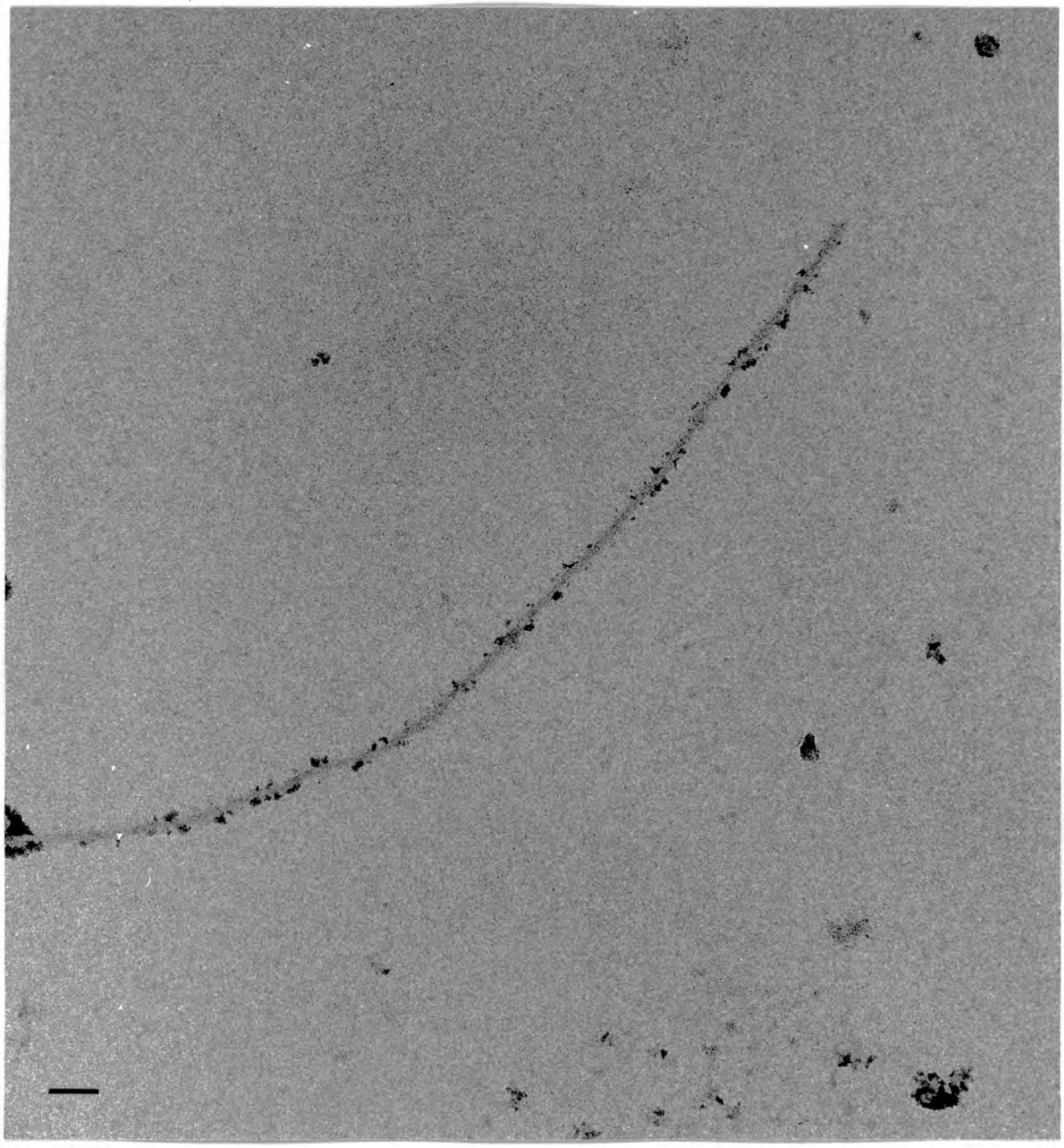


Figure V.1: Electron micrograph of a leaf-dip preparation of doubly infected maize (MDMV-B and CMV-K). The sample was trapped with anti-MDMV-B-ST and anti-CMV-K serum mixed 1:1 and decorated with anti-MDMV-B-ST serum. Viewed at a magnification of 90 000. Bar = 100 nm. (See IX.8.(c) and Chapter VIII, Discussion).

E. EVALUATION OF MAIZE CULTIVARS/HYBRIDS/LINES FOR THEIR SUSCEPTIBILITY TO MDMV-B-ST AND CMV-Y

It was of interest to find maize cultivars which would

i) support replication of the two viruses; MDMV-B-ST and CMV-Y.

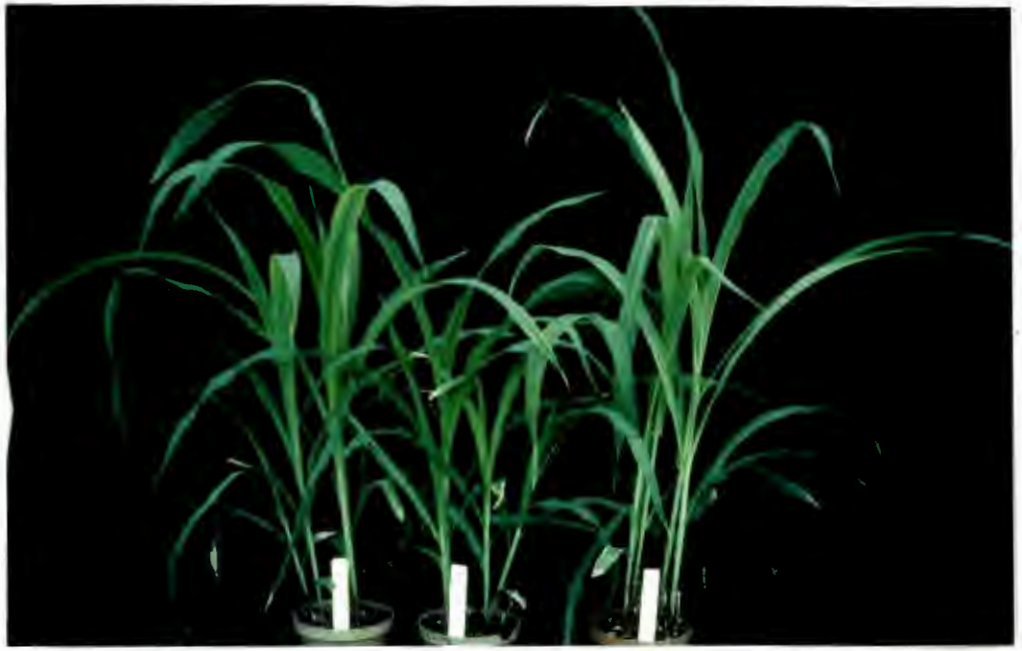
ii) give clear symptom expression

as both these factors were essential in the envisaged programme. In a preliminary screening exercise, a selection of maize breeding lines, cultivars and hybrids was requested from the Summer Grain Center, Potchefstroom (Phase 2, 1983-1984 Programme) to determine whether some of them supported the replication of MDMV and CMV selectively, or whether they were particularly susceptible to one or both of the two viruses.

Maize plants were sap-inoculated with either CMV-Y or MDMV-B-ST. After 10 days they were checked for symptom expression i.e dwarfing, mosaic and compared to uninoculated control plants of each maize line/cultivar/hybrid. Although the results of this exercise gave valuable insight into the susceptibility of maize to the viruses under investigation, details of the full experiment are not presented here. These were reported separately as part of the Maize Virus Progress Report 1984-1985 and 1985-1986.

It is significant to note that without exception CMV-Y had a severe stunting effect on infected plants. The only visible signs of infection were initial necrotic lesions on sap -inoculated leaves; no other symptoms occurred. This would mean that several other tests would have to be carried out to determine the presence of the virus. In contrast CMV-K produced mosaic on many differrent types of maize (Figure IV.1.c). This was an important factor influencing the decision to choose CMV-K for experimental purposes in this thesis (See Chapter IV).

a



b

c

d

Figure V.2.a, b, c and d presents examples of maize infected with MDMV-B-ST and CMV-Y. The four examples chosen more or less represent the reaction observed throughout the 50 maize cultivars: a= PNR 95; b= SNK 2148; c= HL 2; and d= A 1600. In each photograph the plants are arranged from left to right: uninfected maize, infected with CMV-Y, infected with MDMV-B-ST. In most cases, MDMV-B-ST, had little stunting effect on infected plants when compared to uninfected controls; the mosaic symptoms are not clearly visible in the photographs. In contrast CMV, with few exceptions, caused significant stunting of infected plants when compared to control plants.

F. APHID TRANSMISSION STUDIES

1. Introduction

Maize dwarf mosaic virus is transmitted in a non-persistent manner by some 20 aphid species (Louie and Bancroft, 1981). All the species do not have the same efficiency of transmission. Bancroft et al. (1966) tested ten different aphid species and found that Dactynotus species were the most efficient vectors of MDMV. Other aspects of MDMV-B transmission are discussed by Chauhan (1985). Cucumber mosaic virus is also transmitted by many different aphid species. The most common species are Myzus persicae and Aphis gossypii (See Chapter II)

The aphids used in these experiments were obtained from ongoing research programmes in the UCT Microbiology Department (maize virus and wheat virus research programmes). The following aphid species were used

a) Rhopalosiphum padi colony (RhPV negative, maintained by Ms C. Williamson for her research on RhPV)

b) A Rhopalosiphum maidis colony collected from barley plants in the Western Cape and cycled several times over clean barley plants to ensure that they were virus-free

c) A Myzus persicae colony collected from roses in a city garden and maintained on zinnia plants (see Chapter IX.C).

2. Procedure

For aphid transmission experiments the procedure was as follows:

a) Virus acquisition: Three to four aphids were allowed to feed on sap-inoculated plants (approximately 7 days post-inoculation; when symptoms were first noticed indicating that they were positively infected). When doubly infected plants were used as the acquisition host, the top of the maize plant was cut off just above the growth point so that new growth could occur. The sap of the leaves which had been removed, was tested for the presence of both viruses by DAS-ELISA (IX.D.5). Plants which were positive for both MDMV and CMV were kept for aphid feeding. This procedure ensured that new infected tissue was available for aphid acquisition feeding.

R. padi aphids generally prefer to feed on the maize stem whereas R.maidis prefer the young leaf tissue. M. persicae are not normally associated with maize and so their colonizing behaviour was not taken into account. R. maidis and R. padi do not normally colonize squash plants whereas M. persicae readily feeds on this host. The colonizing behaviour of aphids on different hosts may affect the efficieny of aphid transmission (Chapter VIII, Discussion).

b) Inoculation feeding: The aphids were allowed to feed on the acquisition hosts for one to three minutes before being transferred to four day-old maize seedlings or squash at the dicotyledonous stage. Inoculation feeding was 12 hours (overnight). The plants were sprayed with insecticide and then transferred to plant growth rooms. Plants were processed ten days post-inoculation feeding, to assay for the presence of virus.

c) Controls: For controls, virus-free aphids were allowed to feed on uninfected plants and were transferred to uninfected maize or squash plants.

3. Results

Table V.5 lists the various combinations of

i) aphid species

ii) acquisition hosts

iii) inoculation hosts.

An exact timing, in seconds, of acquisition feeding was not performed because it was considered to be irrelevant. The important aspect to be established from this experiment was whether short feeding times (minutes) would allow transmission of single and double infections i.e. whether the mode of transmission was non-persistent for single and double infections. This would simulate natural feeding behaviour considering that hosts, not normally colonized, were used.

Table V.5.a indicates that CMV-K is transmitted non-persistently by all three aphid species tested. It must be noted that although squash is not normally colonized by R. maidis, CMV-K may still be transmitted to this plant by this aphid species. Only a short probe is required for transmission to occur. MDMV-B-ST (Table V.5.b) is clearly non-persistently transmitted by the aphid species. Inefficient transmission occurred with a long acquisition feed (one hour).

Table V.5.a, b and c: Aphid transmission of MDMV and CMV in single and double infections

a) Transmission of CMV-K

Aphid species	Acquisition host[1]	Inoculation host[2]	Acquisition time[3]	No. plants positive[4] / No. plants tested	% infected
R. maidis	CMV-K infected squash	Uninfected squash	1-3 mins	20/46	44%
"	CMV-K infected squash	Uninfected maize-A	1-3 mins	2/15	13%
"	CMV-K infected maize	Uninfected squash	1-3 mins	8/13	61%
"	Uninfected maize	Uninfected maize-A	1-3 mins	0/20	0%
R. padi	CMV-K infected maize	Uninfected maize-A	1-3 mins	2/15	13%
M. persicae	CMV-K infected maize	Uninfected maize-A	1-3 mins	15/20	75%
"	Uninfected maize	Uninfected maize-A	1-3 mins	0/5	0%

b) Transmission of MDMV-B-ST

Aphid species	Acquisition host[1]	Inoculation host[2]	Acquisition time[3]	No. of plants positive / No. of plants tested	% infected
R. maidis	MDMV-B-ST infected maize	Uninfected maize-A	1-3 mins	49/77	64%
"	MDMV-B-ST infected maize	Uninfected maize-A	1 hour	1/28	3%
"	Uninfected maize	Uninfected maize	1-3 mins	0/10	0%
R. padi	MDMV-B-ST infected maize	Uninfected maize-A	1-3 mins	12/20	80%
"	Uninfected maize	Uninfected maize	1-3 mins	0/10	0%
M. persicae	MDMV-B-ST infected maize	Unifected maize	1-3 mins	9/20	45%
"	Uninfected maize	Uninfected maize	1-3 mins	0/10	0%

c) Aphid transmission from doubly infected plants

Aphid species	Acquisition host[1]	Inoculation host[2]	Acquisition time[3]	MDMV-B-ST		CMV-K	
				No. positive / No. tested	% infected	No. positive / No. tested	% infected
R. maidis	Doubly infected maize-A	Uninfected maize	1-3 mins	7/33	21%	2/30	7%
"	Doubly infected maize-A	Uninfected squash	1-3 mins	0/27	0%	18/36	50%
R. padi	Doubly infected maize-A	Uninfected maize	1-3 mins	7/14	50%	4/14	28%
M. persicae	Doubly infected maize-A	Uninfected maize	1-3 mins	2/28	7%	12/28	43%

1. Three to four aphids were allowed to feed on the acquisition hosts.
2. Aphids were left for 12 hours on inoculation host.
3. The exact time in seconds was not recorded.
4. Inoculation host plants were tested by DAS-ELISA using homologous antisera for the presence of virus (V.C).

CHAPTER VI

MAIZE PROTOPLASTS AND THEIR INFECTION

A. ISOLATION OF MAIZE PROTOPLASTS

1. Introduction

There have been a few reports on the isolation of maize protoplasts for the investigation of their various physiological properties. It is well known that the conditions for the production of mesophyll protoplasts vary according to the plant species and the specific cultivar used. With this in mind, a preliminary study on optimizing conditions for protoplast production from the maize cultivars at our disposal was undertaken.

2. Leaf preparation

Seed was pregerminated in moist vermiculite at 30°C for two days. Only vigorously germinating seeds were transplanted into soil and grown at 26°C under VHO Growlux fluorescent light at a 14 h light/10h dark cycle. After 10-12 days the plants had three leaves. The first two leaves were removed from the plant. As far as possible, all work concerning isolation of

protoplasts was performed in a laminar air flow cabinet using sterile equipment and solutions. The maize leaves were surface sterilized by dipping them in a 1:20 dilution of 3,5% sodium hypochlorite (Jik, Reckitt and Colman, South Africa), for three minutes, followed by three washes of three minutes each in sterile diluted water. The abaxial epidermis was peeled off with fine pointed forceps. The peeled leaf was cut into small pieces using a sterile surgical blade and transferred peeled-side downwards into a small conical flask containing 20 ml of enzyme digestion medium (VI.A.3.(a)). All media was filtered through membrane filters of 0,2 um pore size (Sartorius brand, type SM 66).

3. Conditions for the isolation of maize protoplasts

a) Enzyme digestion mixtures and osmoticums

Several combinations of cell-wall digesting enzymes and two different osmoticums, sorbitol and mannitol, at different molarities, were used (see Table VI.1 for details) in an attempt to produce maize protoplasts in high numbers and in an intact state.

The leaves of maize-C (see IX.C) seedlings were prepared as described in VI.A.2. Incubation of the leaves in each of the different digestion

media was carried out in the dark (container covered by cardboard box) on a linear two-way shaker set at a slow speed (third step of 10 possible speed settings) for 2,5-3 hours at 22°C. The digested leaf material was filtered through sterile cheesecloth into glass centrifuge tubes and the protoplasts collected by centrifugation in a bench centrifuge (BHG type) for 1,5 minutes at appromximately 2 000 rpm. (The selected speed was just enough to collect the protoplasts without compacting or disrupting them). The protoplasts were washed by three cycles of centrifugation in the respective osmoticums (Table VI.1). The mean value of three independent counts using a Spencer Neubauer haemocytometer was used to estimate the number of protoplasts obtained. Table VI.1 presents the results obtained using leaves of maize-C seedlings prepared as described in VI.A.2.

Table VI.1: Combinations of digesting enzymes and osmoticums used for production of maize protoplasts from maize-C. Stripped leaves were digested for 3 hours in each mixture.

Digestion mixture	Average No. of protoplasts/ml[5]
A. 0,6 M Sorbitol[1] 5% cellulase[3] 2,5% macerozyme[4]	8×10^4
B. 0,8 M Sorbitol 5% cellulase 2,5% macerozyme	8×10^4
C. 0,7 M Mannitol[2] 1% cellulase 0,05% macerozyme	3×10^5
D. 0,7 M Mannitol 2% cellulase	5×10^5

1, 2, 3 and 4: Materials (IX.A.6).

5: Average of counts of three independent experiments was calculated.

Although not yielding the number of protoplasts quoted in the literature (usually 1 x 10^6 protoplasts/ml) for other maize cultivars, it appeared that 2% cellulase in 0,7 M mannitol was the most suitable for conditions tested for protoplast isolation from maize-C leaves.

b) Isolation of protoplasts from different maize cultivars

It was important to determine which available maize cultivar/hybrid gave the highest yield of protoplasts. In addition, it was hoped that this cultivar/hybrid would be one which was a good host for both CMV and MDMV so that infection studies could be done (see Chapter VIII,Discussion.). It must be remembered that infection of whole plant tissue is often different to that of isolated protoplasts. Furusawa and Okuna (1978) found that Japanese radish plants could not be sap-inoculated with BMV whereas isolated protoplasts became infected. The host range of plant viruses can often be extended by isolating protoplasts from apparently non-susceptible plants.

The leaves of five different maize cultivars were prepared as described in VI.A.2. The digestion medium was 2% cellulase in 0,7 M mannitol. Table VI.2 shows the yields of protoplasts which were produced.

Table VI.2: Protoplast yields from maize-A, -B, -C, -D and KEP[1]. A mean value of counts was calculated from three experiments.

Maize type	Average number of protoplasts per ml
A	2×10^6
B	1×10^6
C	5×10^5
D	2×10^6
KEP	4×10^5

1. See materials (IX.C.)

It was evident that maize-A, -B and -D yielded numbers of protoplasts which neared those reported for cereal plants in the literature (Okuno and Furusawa, 1977; Chin and Scott, 1979; Day et al., 1981). The ease with which the lower epidermis could be peeled varied between different maize types. Maize-A gave the highest yield of protoplasts and had leaves suitable for peeling. Results of laboratory infection of maize with CMV indicated that this maize type was a good host for CMV giving clear expression of infection and easily recognizable symptoms (Figure IV.1.C). For these reasons this maize was chosen for further work.

c) Incubation conditions

The length of time protoplasts are viable in suspension may be extended firstly by incubating them in media containing inorganic ions and anti-contaminants, and secondly in the most suitable light and temperature conditions.

The incubation conditions for protoplasts from maize-A leaves were tested by storing the protoplast preparations in various light and temperature conditions. The incubation medium of Okuno and Furusawa (1978) (IX.A.6.(c)) or alternatively 0,7 M mannitol without inorganic supplements were used for suspension of the protoplasts after their isolation. After 24 hours in different conditions (Table VI.3) the protoplast preparations were counted. The exclusion dye, Evans blue (Gurr, BDH Chemicals) was used to test their viability (IX.12). Table VI.3 shows that when stored in incubation media under continuous light at 22°C, 84% of the maize protoplasts survived after 24 hours.

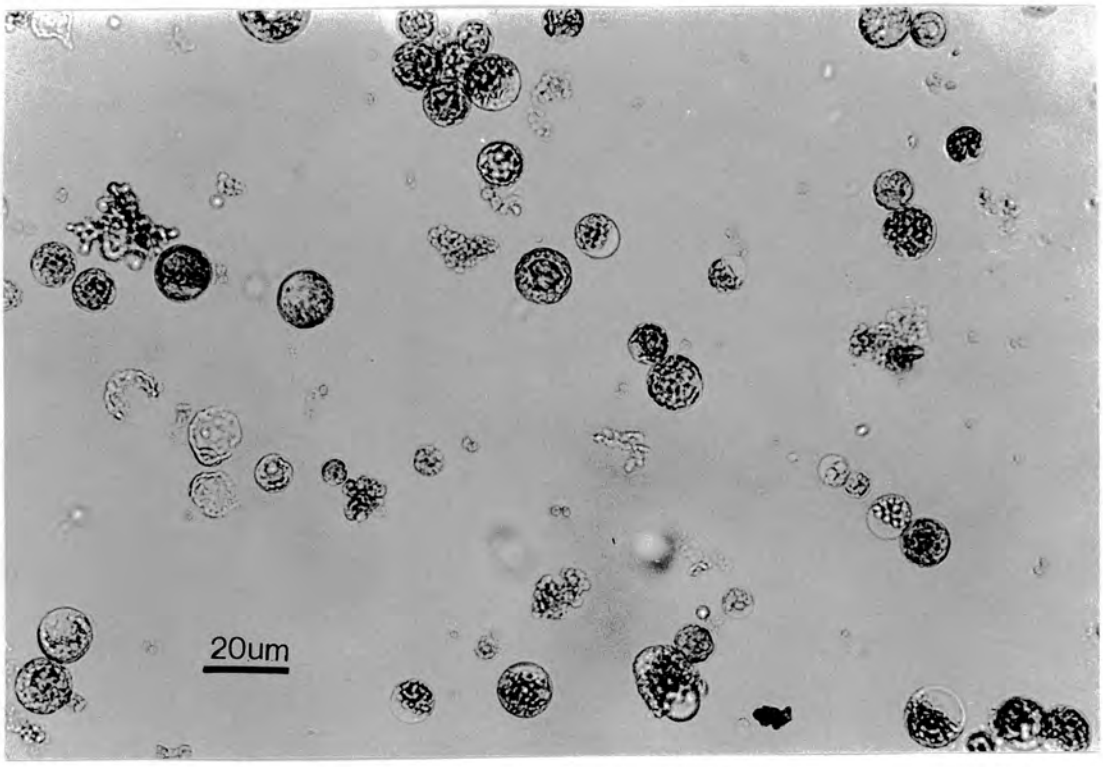
Figure VI.1 shows a population of mesophyll protoplasts isolated from maize-A leaves, viewed at two magnifications.

Table VI.3: Incubation conditions for protoplasts (maize-A)

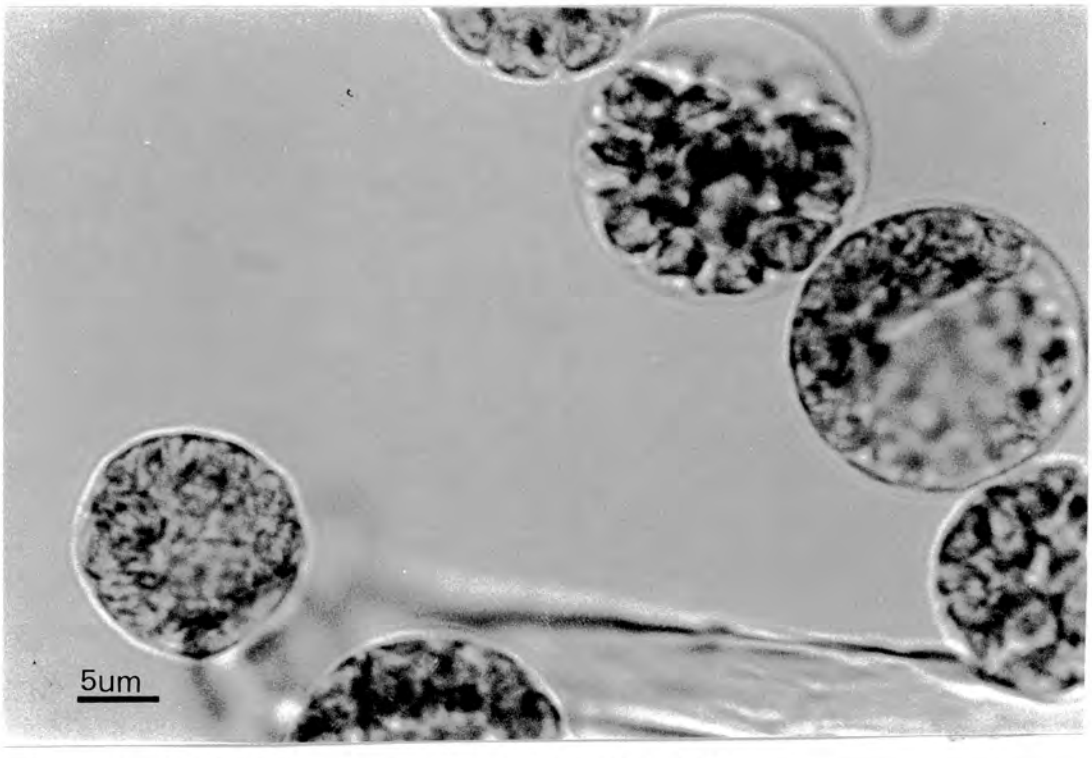
	Conditions	% surviving protoplasts after 24 hours[2]
A	22°C 16 h light/8 h dark[1] in 0,7 M mannitol	23%
B	22°C 16 h light/8 h dark in incubation medium (IX.A.6.(c))	78%
C	25°C/22°C 16 h light/8 h dark in 0,7 M mannitol	38%
D	25°C/22°C 16 h light/8 h dark in incubation medium	58%
E	25°C day/20°C night temperature in plant growth rooms 14 h light/10 h dark in incubation medium	54%
F	22°C Continuous light in incubation medium	84%

1. VHO Growlux tubes fitted with a timer switch were used at 30 cm above the protoplasts.
2. The viability of protoplasts was assessed using Evans Blue (IX.12). Averages of three independent experiments were calculated.

Figure VI.1.a and b: Maize protoplasts isolated from leaves of 10 day old Maize-A seedlings. Protoplasts were isolated by incubating stripped maize leaves in 0,7 M mannitol containing 2% cellulase for 2,5 - 3 hours in the dark at 22°C



a) Viewed at a magnification of 280X.



b) Viewed at a magnification of 720X.

B. INFECTION OF MAIZE PROTOPLASTS WITH BROME MOSAIC VIRUS (BMV-ST)

1. Introducton

Since no information on the infection of maize mesophyll protoplasts with plant viruses was available, it was initially decided to use BMV-ST to establish an infection protocol. This virus had been used previously in the Department to infect barley protoplasts using the method of Okuno et al. (1977). It was hoped that this method could be adapted for inoculation of maize protoplasts with the virus.

2. Method for infection with BMV

Protoplasts from maize-A leaves prepared as described in VI.A.2 and 3 were pelleted by centrifugation, resuspended and pre-incubated for five minutes in 0,02 M sodium citrate (pH5,6) (IX.A.6.(d)) containing 0,7 M mannitol and 2 ug/ml poly-l-ornithine (PLO) (Sigma, M_r= 122 000). Counts of the protoplasts were made and the suspension adjusted with the same buffer to contain $1x10^5$ protoplasts/ml. Since the system was set up to establish the feasibility of infecting maize protoplasts with a plant virus, the effect of virus concentration on the efficiency of

infection was not investigated. It was decided to use 100 ug/ml BMV-ST since this was the concentration used by Okuno et al. (1977). BMV-ST in 0,02 M sodium citrate (pH 5,6) was added at 100 ug/ml to this suspension. The mixture was incubated for 10-15 minutes on a linear two-way shaker set at a slow speed. Alternatively the virus/protoplast mixture was left stationary on the bench with occassional gentle swirling. Following incubation with the virus the protoplasts were collected by bench centrifugation, washed three times in 0,7 M mannitol (IX.A.6.(b)) and the final protoplast pellet resuspended in incubation medium (IX.A.6.(c)). For controls protoplasts from maize-A leaves were mock-inoculated i.e. treated as described above with no virus. The uninfected protoplasts were kept at 22°C under continuous cool white fluorescent light.

3. Detection of BMV in maize protoplasts

Before determining whether the protoplasts were infected with BMV, they were incubated for 24 hours (Table VI.3.F). The uninfected and BMV-infected protoplasts were washed by three cycles of centrifugation and resuspension in 0,7 M mannitol. The final pellet of protoplasts was resuspended in 100 ul of dissociation mix (IX.A.4.(e)) for disruption of virus (if present) , subjected to PAGE gel electrophoresis

(IX.D.6) and immuno-electroblotting (IEB) (IX.D.7). Initially IEB was chosen because of its sensitivity. Anti-BMV serum was used to probe the electroblot. Figure VI.2 is an immuno-electroblot of disrupted BMV-infected protoplasts. The presence of BMV is clearly visible in lane 1, which contained the disrupted,infected maize protoplasts. The faint reaction in lane 3 indicates that some BMV (or BMV protein) remained in the supernatant fluid after washing the protoplasts. This could have been caused either by virus particles which had not passed through the protoplast membrane or had not adsorbed to its surface. No reaction was obtained with mock-inoculation protoplasts (lane 2).

Since the positive reaction obtained with BMV-infected protoplasts and anti-BMV serum could be the result of the virus particles merely adhering to the outside of the protoplasts, one cannot confidently report from the results of IEB that BMV had infected the maize protoplasts.

4. Replication of BMV in maize protoplasts

Maize protoplasts were prepared, inoculated and incubated as before. After 12 and 36 hours post-inoculation, samples of 1 x 10^5 protoplasts/ml were washed in 0,7 M mannitol and homogenized using a Polytron (Kinematica GM 6H) fitted with a fine probe (0,8 cm in diameter). They were kept frozen until required for testing by DAS-ELISA (IX.D.5).

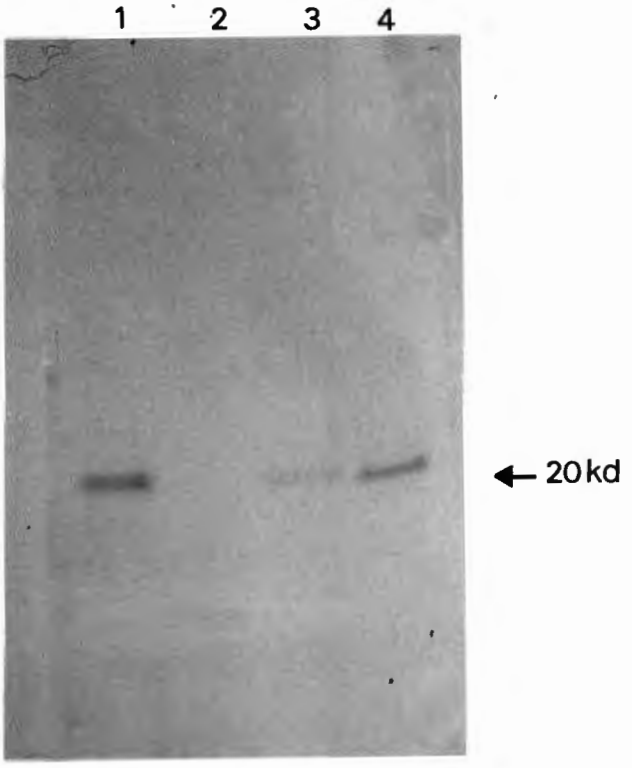


Figure VI.2: Immuno-electroblot of protoplasts infected with BMV-ST and incubated for 24 hours. Anti-BMV serum at a 1/30 dilution used to probe the electroblot. Note the BMV protein in the protoplasts (20 kd; lane 1).

Lane 1 - Protoplasts infected with BMV-ST

2 - Uninfected, mock-inoculated protoplasts

3 - Supernatant atter third wash of infected protoplasts

4 - BMV-ST positive control

Table VI.4 shows the results when the homogenates of the infected protoplasts after 12 and 36 hours of incubation were reacted with anti-BMV IgG and conjugate.

Table VI.4: Results of DAS-ELISA of protoplast extracts 12 and 36 hours after inoculation with 100 ug/ml BMV-ST.

Time of Incubation (hours)	Absorbance at 405 nm[4]			
	Infected[1]	Uninfected[1]	Supernatant[2]	BMV-ST[3]
12	0,233	0,070	0,121	1,893
36	0,962	0,030	0,174	1,688

1. Protoplasts were washed by centrifugation and homogenized as described VI.B.4. Readings indicated at a 1/4 dilution of the homogenates in post-coating buffer (IX.A.3.(c)).
2. The supernatant after the third wash of the infected protoplasts was tested.
3. BMV-ST (4 mg/ml) was used as a positive control at a 1/1 000 dilution in post-coating buffer.
4. An average of two readings of absorbance at 405 nm is indicated. Anti-BMV IgG and conjugate were used at a 1/500 dilution.

The results in Table VI.4 show a definite increase in the quantity of BMV present in the disrupted protoplasts after 36 hours of incubation when compared to protoplasts incubated for only 12 hours. This would indicate that the virus was replicating within the protoplasts. The supernatants after the third washing cycle of the infected protoplast samples were also tested. A weak positive reaction occurred. This infers that some virus particles were present possibly as a result of the disruption of protoplasts during the procedure and also due to aging (see VI.H).

The BMV-infected protoplasts after 36 hours of incubation were serially diluted in PBS-T-BSA (IX.A.3.(c)) and titrated against a known concentration of BMV-ST (4 mg/ml), The concentration of BMV-ST in the protoplasts was calculated to be 0,9 mg/ml.

5. Time course of BMV infection of maize protoplasts

Once it had been shown that BMV could replicate in the maize protoplasts, a time course study could be undertaken. Infected protoplasts (1×10^5/ml) were collected, washed and homogenized after 0, 2, 3, 12, 21 and 24 hours of incubation after inoculation. The samples were tested for the presence of BMV by DAS-ELISA (IX.D.5). Figure VI.3 shows the infection curve obtained. Multiplication of the virus is indicated by the increase in the readings of absorbance at 405 nm with increasing time of incubation. After 24 hours it appeared that the plateau of the infection curve had been reached. No reaction was obtained with mock-inoculated protoplasts.

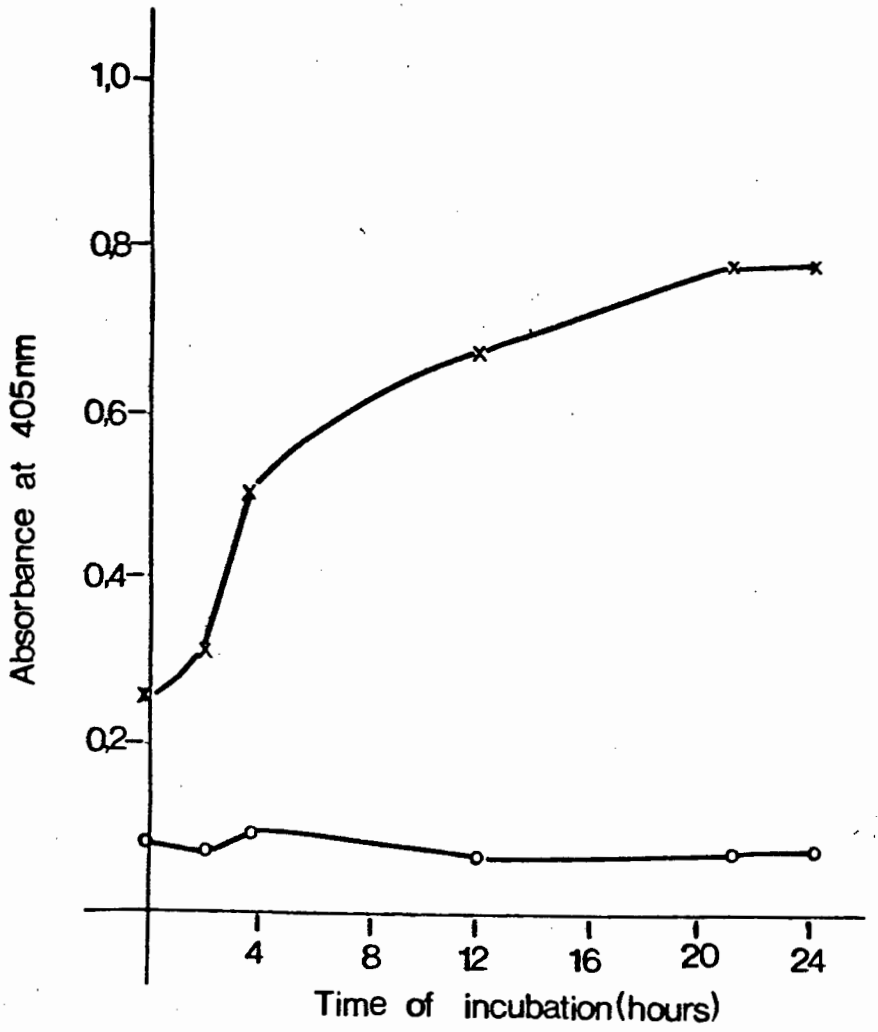


Figure VI.3: Graph to show time course of infection of maize-A protoplasts with BMV-ST. Protoplasts were inoculated with 100 ug/ml virus in the presence of 2 ug/ml PLO. Samples were assayed by DAS-ELISA 2, 3, 12, 21 and 24 hours after inoculation (x—x).

Anti-BMV IgG and conjugate were used at a 1/500 dilution. Control was uninfected protoplasts which had been mock-inoculated and were also sampled and tested (o—o). Readings plotted are the average of three independent experiments.

C. INFECTION OF MAIZE PROTOPLASTS WITH CUCUMBER MOSAIC VIRUS

1. Introduction

Although there have been several reports of infection of mesophyll protoplasts with CMV (Table II.4), no references could be found of CMV inoculation of maize protoplasts. As already mentioned, CMV is not normally associated with maize but may often be overlooked because of its insidious nature. In addition it had been shown to occur together with MDMV in field collected maize (Chapter III). It was therefore of interest to study CMV infection of maize protoplasts. This would perhaps provide some insight into the infection of maize with CMV and its interaction with other viruses, in particular MDMV.

2. Method for infection of protoplasts with CMV-K

a) Concentration of CMV-K

CMV-K was initially selected for infection since it had been previously reported that it could infect maize (Rao and Francki, 1981; Tien Po *et al.*, 1982). In addition, earlier infection studies showed that this strain readily infected maize-A plants (Chapter IV). CMV-K was maintained in glutinosa tobacco but inoculated on to squash seedlings for virus extraction (IX.D.1). For infection of protoplasts, various concentrations of

virus in the inoculum had been reported (Table II.4). Maize protoplasts were isolated as described previously. CMV-K was preincubated at various concentrations i.e. 10, 50, 100 and 150 ug/ml for five minutes in 9 ml of 0,02 M potassium phosphate buffer (pH 5,8) (IX.A.1.(a)) containing 0,7 M mannitol and 2 ug/ml PLO. After this incubation time, the mixture was carefully added to the protoplast pellet, the protoplasts were gently resuspended and left for 15-20 minutes with occassional gentle shaking. Washing and resuspension in incubation medium was carried out as before (VI.A.3.(c)). The protoplasts were left for 24 hours at 22^{o}C under continuous light before testing by DAS-ELISA (IX.D.5). Figure VI.4 reveals that 50 ug/ml of CMV-K in the inoculum was optimum for infection of protoplasts. The relatively high concentration of CMV-K which was required to obtain optimal infection, is in contrast to other reports which give a concentration of 2 ug/ml for most CMV strains, when infecting protoplasts from other plant species. (See Figure IV.2.(a) and (b) and Chapter VIII, Discusssion, for explanation).

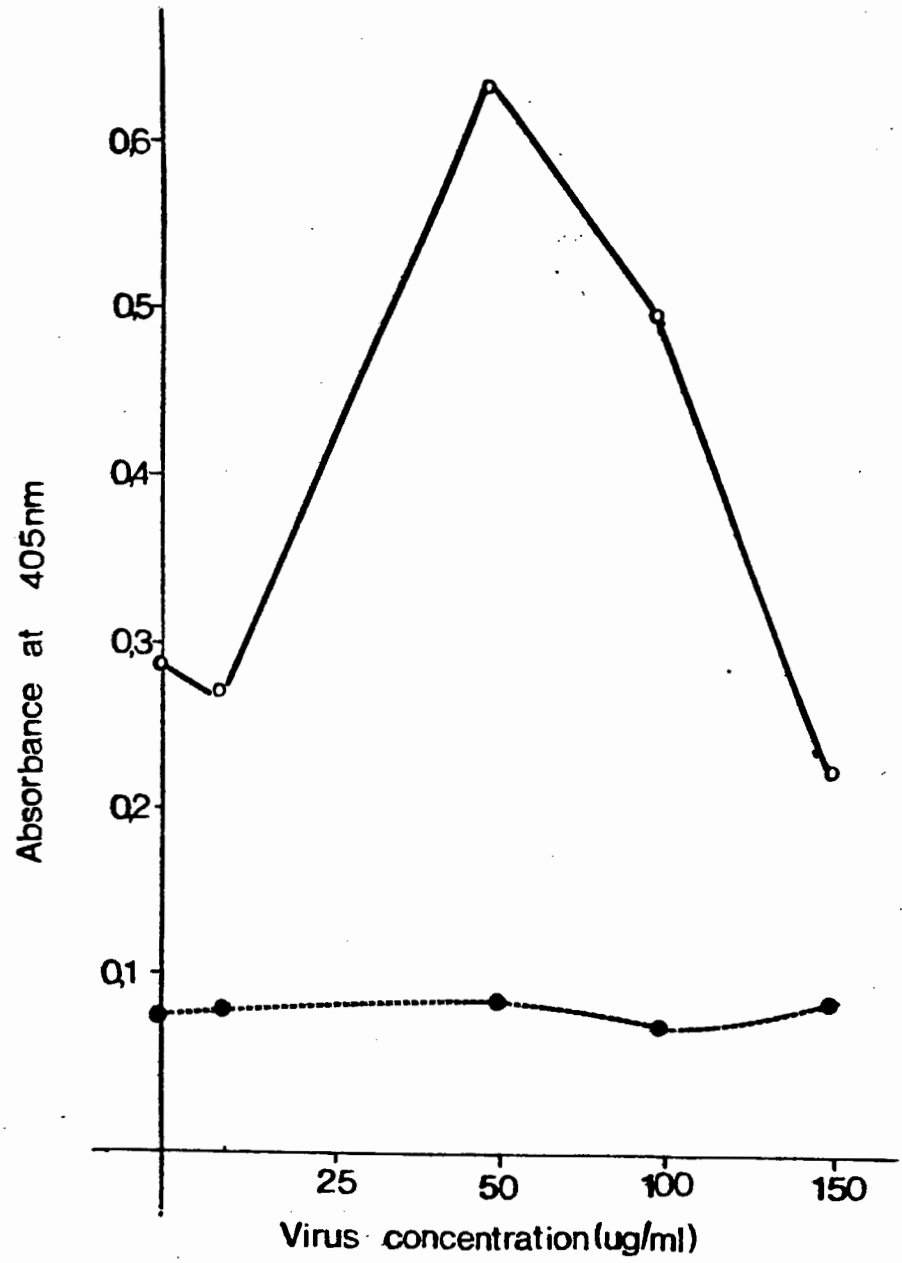


Figure VI.4: Graph to show the effect of varying concentrations of CMV-K in the inoculum. Protoplasts (1 x 10^5/ml) were assayed for the presence of virus by DAS-ELISA after 24 hours of incubation. An average of three readings is plotted. Anti-CMV-K Ig G and conjugate were used at a 1/300 dilution.

(o—o) = Protoplasts infected with varying concentrations of CMV strain K.

(●---●) = Uninfected protoplasts.

b) The necessity for Poly-L-ornithine

Poly-L-ornithine (PLO), a polycation, is often required for successful infection of protoplasts. The necessity for PLO varies for different viruses. For BMV, PLO is not essential for infection to occur, although its presence enhances the efficiency of infection (Okuno and Furusawa, 1978). For TMV, infection of protoplasts does not occur without it (Takebe and Otsuki, 1969).

It was necessary to investigate whether PLO was critical for infection of maize protoplasts with CMV. Protoplasts were isolated and infected with CMV-K as described previously except that PLO was omitted from the inoculum in one case and included in another. A concentration of 2 ug/ml PLO was used in the latter. Table VI.5 indicates that protoplasts which had been inoculated without PLO showed only a background reaction when tested after 24 hours by DAS-ELISA. However a significant amount of CMV-K could be detected in the protoplast sample which had been in contact with PLO during inoculation.

Table VI.5: The effect of PLO on infection of maize protoplasts with CMV-K.

PLO Treatment	Absorbance at 405 nm[3]
Protoplasts infected in[1] the presence of PLO[2]	0,581
Protoplasts infected in the absence of PLO[2]	0,243
Uninfected protoplasts No PLO	0,080

1. Protoplasts were inoculated with 50 ug/ml CMV-K (VI.C.2) and incubated for 24 hours.
2. The virus was preincubated either in the presence or absence of PLO (2 ug/ml; M_r = 122 000).
3. Protoplasts (1 x 105/ml) were homogenized and tested by DAS-ELISA (IX.D.5). Anti-CMV-K IgG and conjugated were used at a 1/300 dilution. Averages of two readings for three separate experiments are indicated (see Discussion).

3. Time course of infection of maize protoplasts with different strains of CMV.

CMV-K was not the only strain of CMV that could infect maize. Evidence was available that CMV-Y, CMV-Lupin-K5 and CMV-S could also infect maize although symptom expression was limited to necrotic lesions on sap-inoculated leaves (Chapter IV this thesis; P. Lupuwana, 1985; Von Wechmar, unpublished results). It was therefore decided to examine the ability of other CMV isolates to infect maize protoplasts.

CMV-K, CMV-Y and CMV-S, all propagated in squash were used for inoculation at a concentration of 50 ug/ml in the presence of 2 ug/ml PLO as described in VI.C.2. Infected protoplast samples together with mock-inoculated protoplasts (1×10^5 protoplasts/ml) were taken at various time intervals after inoculation. The protoplast samples were frozen until required and then thawed and homogenized for testing by DAS-ELISA. The experiment was carried out on three separate occassions and averages of the absorbance readings at 405 nm for each sample plotted (Figure VI.5).

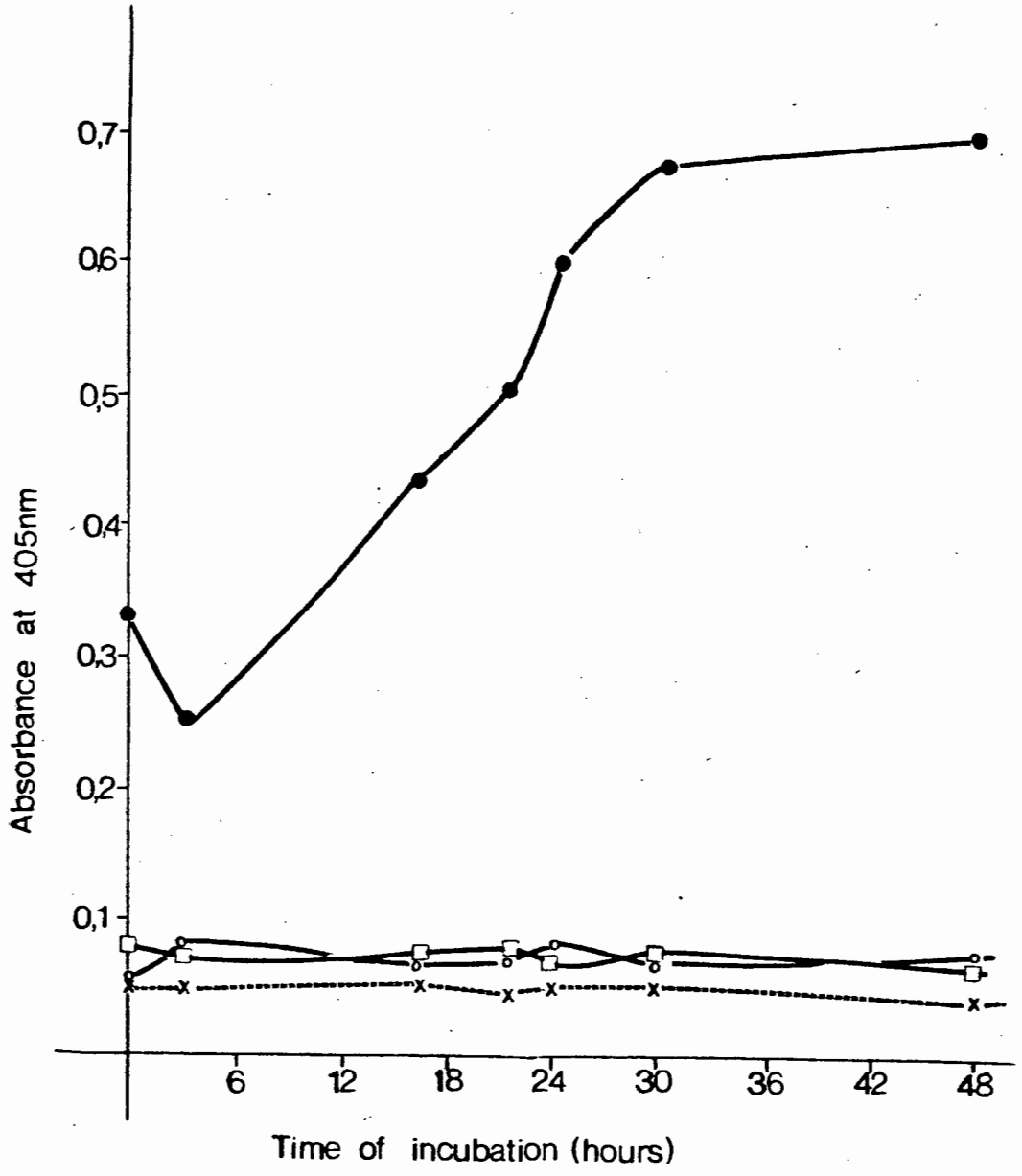


Figure VI.5: Graph to show time course of infection of maize protoplasts with 50 ug/ml of CMV-K (●—●), CMV-S (□—□) and CMV-Y (○—○). Samples of 1 x 10^5 protoplasts were assayed by DAS-ELISA after various times of incubation. Uninfected, mock-inoculated protoplasts indicated by (x····x). Anti-CMV Ig G and conjugate were used at a 1/300 dilution.

The results obtained clearly show that CMV-K replicates within the maize protoplasts whereas CMV-S and CMV-Y were not detected in the inoculated protoplasts. The infection curve of CMV-K is similar to that obtained by Okuno et al. (1977) for infection of barley protoplasts with BMV. After 4 hours of incubation, the virus has begun to multiply within the protoplasts and continues to do so until approximately 30 hours post-inoculation. After this there was no further detectable increase in the amount of virus within the protoplasts. CMV-S and CMV-Y, in contrast,did not infect the maize protoplasts. Mock-inoculated protoplasts only gave background readings in all cases. Because this was an unexpected result, five repeat experiments were performed all of which showed the same tendency. CMV-S and CMV-Y were not used for further work with maize protoplasts.

4. Fluorescent-antibody labelling of infected protoplasts

a) Introduction

The detection of virus particles within protoplasts by fluorescent-labelled antibodies was devised by Otsuki and Takebe (1969) to visualise TMV in isolated protoplasts. The most commonly used fluorescent dye for labelling is fluorescein isothiocyanate (FITC). There are two methods for staining infected protoplasts:

i) using fluorescent labelled gamma globulins prepared against the virus (the direct method),

ii) using rabbit antiserum to the virus and then fluorescent-labelled anti-rabbit serum (the indirect method).

The method was adapted for use with CMV-infected maize protoplasts and is described below.

b) Conjugation of gamma globulins with FITC.

Purified gamma globulins (IX.D.4.) were labelled with the fluorescent dye, fluorescein isothiocyanate (FITC, Merck) according to the method of Otsuki and Takebe (1969). The method is summarized below.

IgG was extracted by ammonium sulphate precipitation (IX.D.4.(a) and (b)). The purified IgG, after the second precipitation, was resuspended in 0,02M sodium carbonate buffer pH 9,8 (IX.A.7.(a)), and adjusted to 4mg/ml. The extinction coefficient of $E^{0,1\%}_{280\ nm} = 1,4$ was used (Clark and Adams, 1977). Two millilitres of this IgG was dialysed against 100 ml of a 0,006% (w/v) FITC solution in 0,02 M sodium carbonate buffer, pH 9,8 (IX.A.7.(a)). Dialysis was carried out using Spectropor No. 4 dialysis membrane tubing (Spectrum

Medical Industries, Inc) at 4°C overnight. The contents of the dialysis bag were passed through a Sephadex G-25 column (Pharmacia Fine Chemicals, Uppsala, Sweden) to free the conjugated globulin from the uncoupled dye. The conjugated antibodies were eluted from the column with 0,02 M sodium carbonate buffer (pH 9,8). One millilitre fractions were collected from the column using an Isco Fraction Collector (Model 320). A Beckman Model 25 Spectrophotometer was used to read the optical density of each fraction at 280 nm and 495 nm.

The first fraction showing high absorbance readings at both 495 nm and 280 nm were collected and pooled. The conjugate was dialysed overnight at 4°C against 0,02 M sodium carbonate buffer, pH 9,8. The conjugated antibodies were absorbed with acetone extracted powder of healthy maize leaves to remove IgG which reacted with maize host proteins. Six grams of leaves were homogenized in a pestle and mortar with 50 ml of acetone. The excess acetone was poured off and the ground leaves left to dry in a fume cupboard fitted with a fast extractor fan. The resulting powder was extracted just before use with 50 ml of 80% ethanol in a water bath set at 80°C for 10 minutes and then washed by three washes with PBS buffer, pH 7,0

(IX.A.1.(a)); each wash for 15 minutes. Two millilitres of conjugated gamma globulins were diluted to twenty millilitres with PBS buffer and added to 0,3 grams of the washed powder. The mixture was stirred for one hour at 22°C and centrifuged at 10 000 rpm for 20 minutes to remove insoluble material.

The fluorescein-protein (F/P) ratio of the conjugates could be calculated according to the equation devised by Holbrow and Johnson (1967).

$$F/P = 0,41 \times \frac{[FITC] \text{ in mg/ml}}{[IgG] \text{ in mg/ml}}$$

where [FITC] ug/ml =

$$\frac{OD_{495\ nm} - 1/2\ OD_{320\ nm} \times \text{dilution factor}}{0,2}$$

The F/P ratio of anti-CMV conjugate was calculated as 0,83. This ratio was within the range of F/P ratio for conjugates that give rise to specific staining (Holbrow and Johnson, 1967).

c) Staining of protoplasts

Protoplasts were isolated and infected with 50 ug/ml CMV-K as described in VI.C.2. After 24 hours of incubation the protoplasts were washed by

centrifugation and resuspended in 0,7 M mannitol. The direct method of FITC-labelled antibody staining was used. A drop of the thick protoplast suspension was smeared on to a clean glass slide and dried quickly in a stream of warm air. (The slides were cleaned with commercial methanol and coated with a 0,1% (v/v) ovalbumin solution (IX.A.7.(c)). Drying the slides in warm air prevented the formation of mannitol crystals which occurred when the slides were air dried and caused the protoplasts to disrupt. The protoplasts were fixed by immersing the slides in acetone for 45 minutes, equilibrated in PBS buffer, pH 7,0 (IX.A.1.(b)) for 90 minutes and then in PBS buffer containing 0,05% BSA for 15 minutes. Excess buffer was drained using Whatman No. 3 filter paper. A few drops of a 1/256 dilution of FITC-conjugated anti-CMV IgG (VI.4.b) were added to the fixed protoplasts and the slides were incubated for 2 hours at 22^{o}C in a humid box. Slides were washed in PBS buffer (pH 7,0) for two hours with three changes of buffer. They were gently drained to remove excess buffer and mounted in carbonate glycerol mounting fluid (IX.A.7.(d)). The specimens were observed using a Zeiss IM 35 microscope fitted with a HBO 50W high pressure mercury source and a 12V 100W halogen source. Barrier filter LP520 and exciter filters BP 450-490 were used. Photographs taken with a Contax RTS camera fitted with an automatic exposure control.

d) Results

CMV-infected protoplasts were successfully labelled with FITC-labelled antibodies (Figure VI.6.a). However, healthy uninfected protoplasts which had been stained in the same way as the infected ones, showed high background fluorescence (Figure VI.6.b).

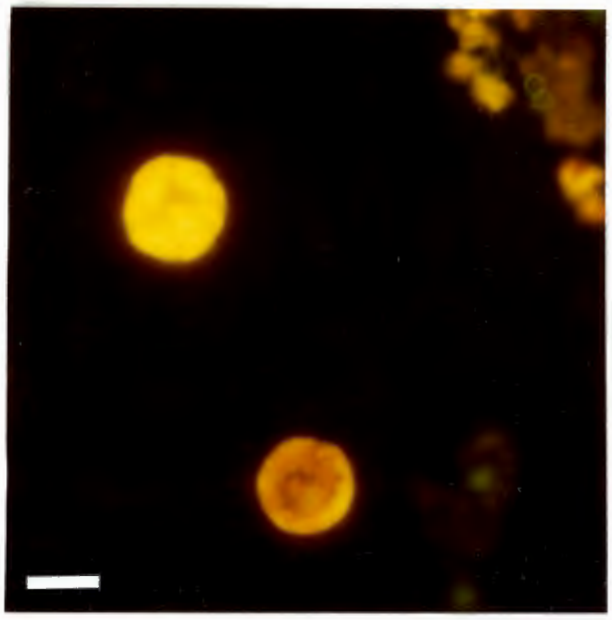
To overcome this, ethanol fixation of the protoplasts to the slide was attempted since it had previously been found that this reduced the background fluorescence in cowpea protoplasts infected with CMV (Koike et al., 1977). Oksuki and Takebe (1973) reported that ethanol caused dispersion of the CMV antigen in tobacco protoplasts. Ethanol fixation of CMV-infected maize protoplasts was not successful as they burst and were destroyed. As an alternative, more stringent absorption of the antisera with host proteins was carried out before FITC conjugation. This was done as follows: Anti-CMV serum was host absorbed, IgG was prepared by ammonium sulphate precipitation (IX.D.4.(b)), the final product after dialysis was adjusted to a concentration of 4 mg/ml and then labelled with fluorescein isothiocyanate. It was further treated with acetone-dried healthy maize extract to remove any remaining antibodies to maize host proteins. This latter step had been

omitted previously. In addition the length of time of washing in PBS buffer after staining was increased from 30 minutes to 90 minutes with several changes of buffer. One wash of 15 minutes in PBS buffer containing 0,05% BSA was included. Figures VI.6.(c) and (d) indicate that the uninfected protoplasts now only exhibited a very low background fluorescence and infected ones fluoresced brightly. Approximately 50% of the infected protoplasts showed fluorescence after 24 hours of incubation. Yellow fluorescence was found in aggregates around the chloroplasts of infected protoplasts after 24 hours. When infected protoplasts were stained and viewed after 3 hours of incubation, only 3-5% were fluorescing.

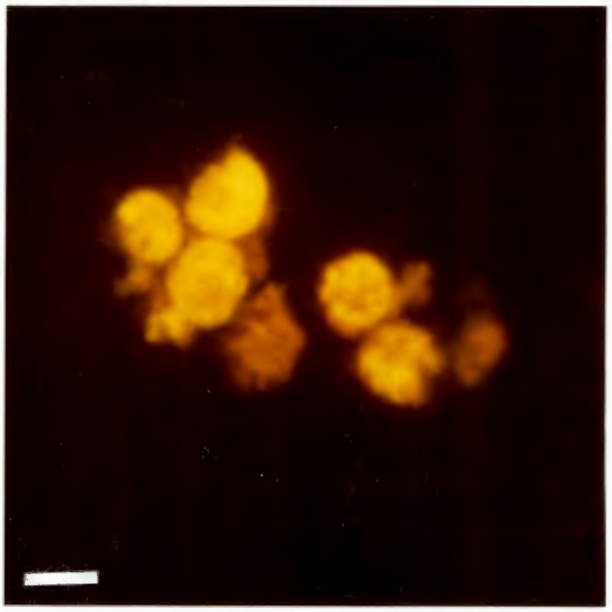
5. Radiolabelling of maize protoplasts

a) Introduction

Analysis of viral-induced proteins and the sequence of their production in infected protoplasts is generally achieved by labelling the protoplast proteins with radioactive amino acids which become incorporated into the protoplast metabolism (Chapter II). It was hoped that by using ^{35}S-methionine, the 24,5 kd CMV protein would become radioactively labelled and could be detected in infected protoplast extracts by polyacrylamide gel electrophoresis and autoradiography.



a



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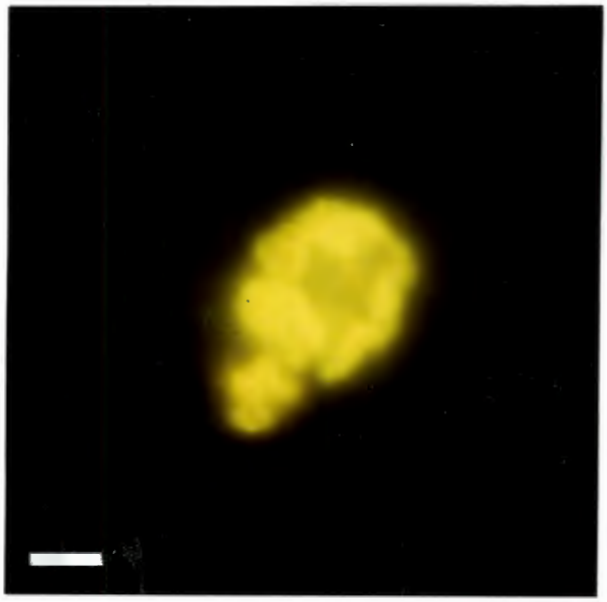

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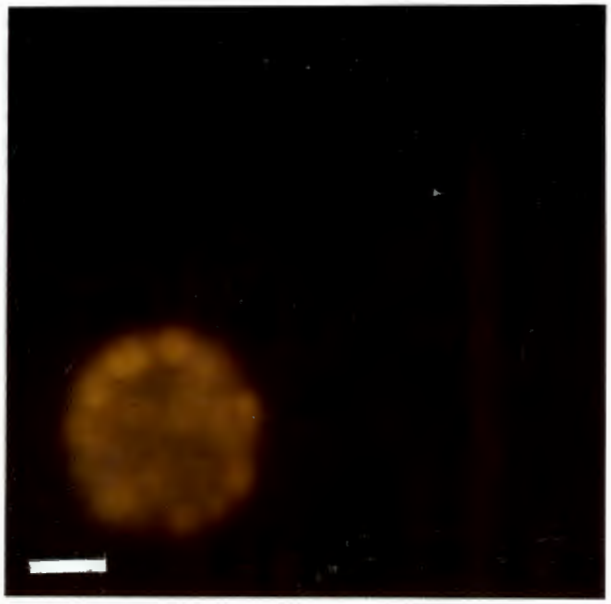


d

Figure VI.6.a, b and c: Fluorescent microscope photographs of maize protoplasts treated with fluorescent-labelled antibodies.

a) Maize protoplasts infected with CMV-K as described in VII.C.2. After 24 hours of incubation, the protoplasts were stained with FITC-labelled anti-CMV IgG at a 1/256 dilution in PBS (VI.C.4.a and b). Magnification was 280x. Bar = 15 um.

b) Uninoculated maize protoplasts stained with FITC-labelled anti-CMV-K IgG at a 1/256 dilution. Note high background auto-fluorescence and non-specific fluorescence. Magnification was 280x. Bar = 15 um.

c) Improved fluorescent labelling of CMV-infected protoplasts (see VI.C.5.d). Magnification was 720x. Bar = 8 um.

d) Uninoculated protoplasts stained with FITC-labelled anti-CMV IgG showing minimal non-specific staining. Magnification was 720x. Bar = 8 um.

b) Incorporation of radiolabel

Maize protoplasts were isolated and infected with CMV-K as described in VI.C. Before incubation, the infected and uninfected protoplasts were divided into two millilitre aliquots in standard containers. The protoplasts were labelled by addition of 20 uCi/ml of L-^{35}S methionine, which was in aqueous solution,containing 0,1% 2-mercapto-ethanol (Amersham International Pty.). The estimated activity of the ^{35}S methionine was 17,36 mCi/ml. The radiolabel was diluted so that it has a final activity of 2uCi/ml. Twenty microlitres of radioactive label was added to two millilitres of protoplast suspension immediately after the protoplasts had been infected with CMV-K. At various times after inoculation, the labelled protoplasts were sampled by washing by centrifugation and resuspension in 0,7 M mannitol as described previously. After the final centrifugaton step, the protoplast pellet was disrupted by heating at 100^{o}C for five minutes in 100 ul of dissociation mixture (IX.A.4.(e)). The samples were stored at 4^{o}C until required for polyacrylamide gel electrophoresis (IX.D.6) and autoradiography(IX.D.11).

c) Results

The abundance of labelled protoplast proteins obscured the detection of CMV-induced proteins in infected protoplasts (Figure VI.7). This may be minimized by irradiation of the protoplasts with UV light (Sakai and Tekebe, 1974). An attempt to do this was made, but the protoplasts were destroyed i.e. no proteins were subsequently produced. Due to time limitation it was decided not to pursue further aspects of this work. The experience gained in handling radioactivity was useful, and led to a greater appreciation of published data in this field.

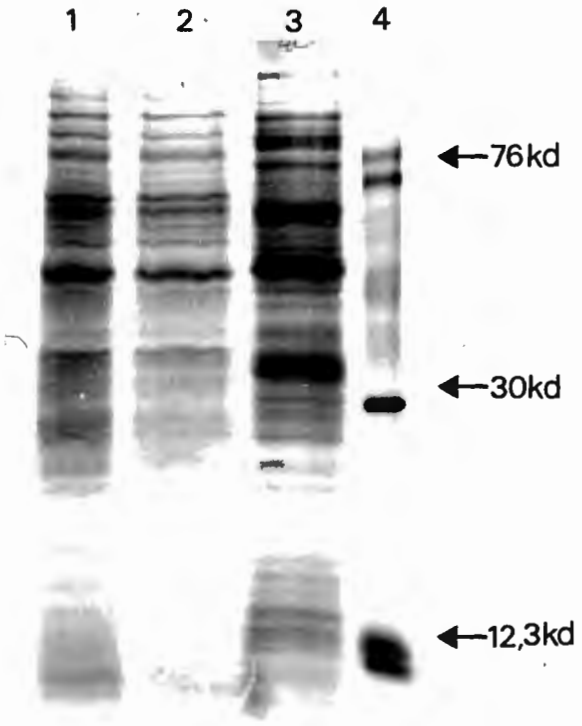


Figure VI.7: Autoradiograph of maize protoplasts labelled with ^{35}S methionine (VI.C.5). The protoplasts were infected with CMV-K (VI.C.2), labelled and incubated for 24 hours. They were disrupted, subjected to PAGE gel electrophoresis (IX.D.6) and then autoradiography (IX.D.11)

Lane 1 - Uninfected protoplasts after 24 hours incubation

2 - Protoplasts infected with CMV-K after 0 hours incubation

3 - Protoplasts infected with CMV-K after 24 hours incubation

4 - M_r marker (Electran, BDH Chemicals); ovotransferrin 76 kd; carbonic anhydrase 30 kd; cyctochrome c 12,3 kd.

D. INFECTION OF MAIZE PROTOPLASTS WITH MAIZE DWARF MOSAIC VIRUS (MDMV-B-ST)

1. Introduction

Few studies report successful infection of plant protoplasts with filamentous viruses. This could possibly be attributed to their flexuous nature which sterically hinders their penetration of the protoplast plasma membrane. Xu et al. (1984) reported that they had infected tobacco protoplasts with a potyvirus (tobacco vein mottling virus, TVMV; see Chapter II, Discussion).

The challenge therefore was to check experimentally whether this was possible by using maize protoplasts and MDMV-B-ST. Having shown that maize protoplasts could be infected successfully with CMV-K (Chapter VI.C), it was also of interest to check whether both CMV-K and MDMV-B-ST could infect maize protoplasts simultaneously.

2. Method for inoculation of protoplasts with MDMV-B-ST

The procedure for inoculating maize protoplasts with MDMV-B-ST initially was the same as for CMV-K (VI.C.2). The maize protoplasts were isolated and infected with 50 ug/ml MDMV-B-ST (purified as in

IX.D.3.(b)) by the method outlined in VI.C.2. The protoplasts were sampled at intervals during 48 hours of incubation after inoculation and assayed by DAS-ELISA (IX.D.5). No increase in the amount of virus with increasing time of incubation was evident (results not presented).

In another attempt, the procedure for tobacco vein mottling virus (Xu et al., 1984) was used for MDMV-B-ST. Their method involved washing the isolated tobacco protoplasts with 200 mM $CaCl_2$ immediately after their inoculation with 1 ug/ml TVMV in the presence of 1 ug/ml PLO. Calcium chloride is known to destabilize protoplast membranes. When maize protoplasts were subjected to washing with 0,7 M mannitol containing 200 mM $CaCl_2$ they were destroyed by the treatment. From this it was concluded that the method of Xu et al. (1984) had to be modified for infection of maize protoplasts with MDMV-B-ST.

This was done in the following manner: MDMV-B-ST was purified by differential centrifugation. Precipitation with polyethylene glycol was omitted as it was thought that this could possibly interfere with the infection process. Purified MDMV-B-ST at a concentration of 2 ug/ml was pre-incubated in 0,02 M potassium phosphate buffer (pH 5,6) (IX.A.1.(a))

containing 0,7 M mannitol and 2 ug/ml PLO for five minutes. Isolated maize protoplasts (VI.A.2 and 3) were collected by centrifugation and resuspended in the inoculum. They were left for 15-20 minutes with occasional gentle shaking. The protoplasts were washed once with 0,7 M mannitol containing 100 mM $CaCl_2$ and then twice in fresh 0,7 M mannitol, before final resuspension in incubation media (IX.A.6.(c)). Samples (1×10^5 protoplast/ml) were assayed for the presence of MDMV-B-ST, over a 66 hour incubation period, by DAS-ELISA as described previously.

3. Results

Figure VI.8 shows the infection curve which was plotted using averages of readings obtained in three separate experiments. After 15 hours the levels of MDMV-B-ST in the protoplasts begin to increase. By 43 hours post-inoculation, multiplication of the virus appeared to have stopped.

Inoculation with higher concentrations of MDMV-B-ST (5 ug/ml and 10 ug/ml) followed the same trend as shown in Figure VI.8 and did not enhance infection of maize protoplasts.

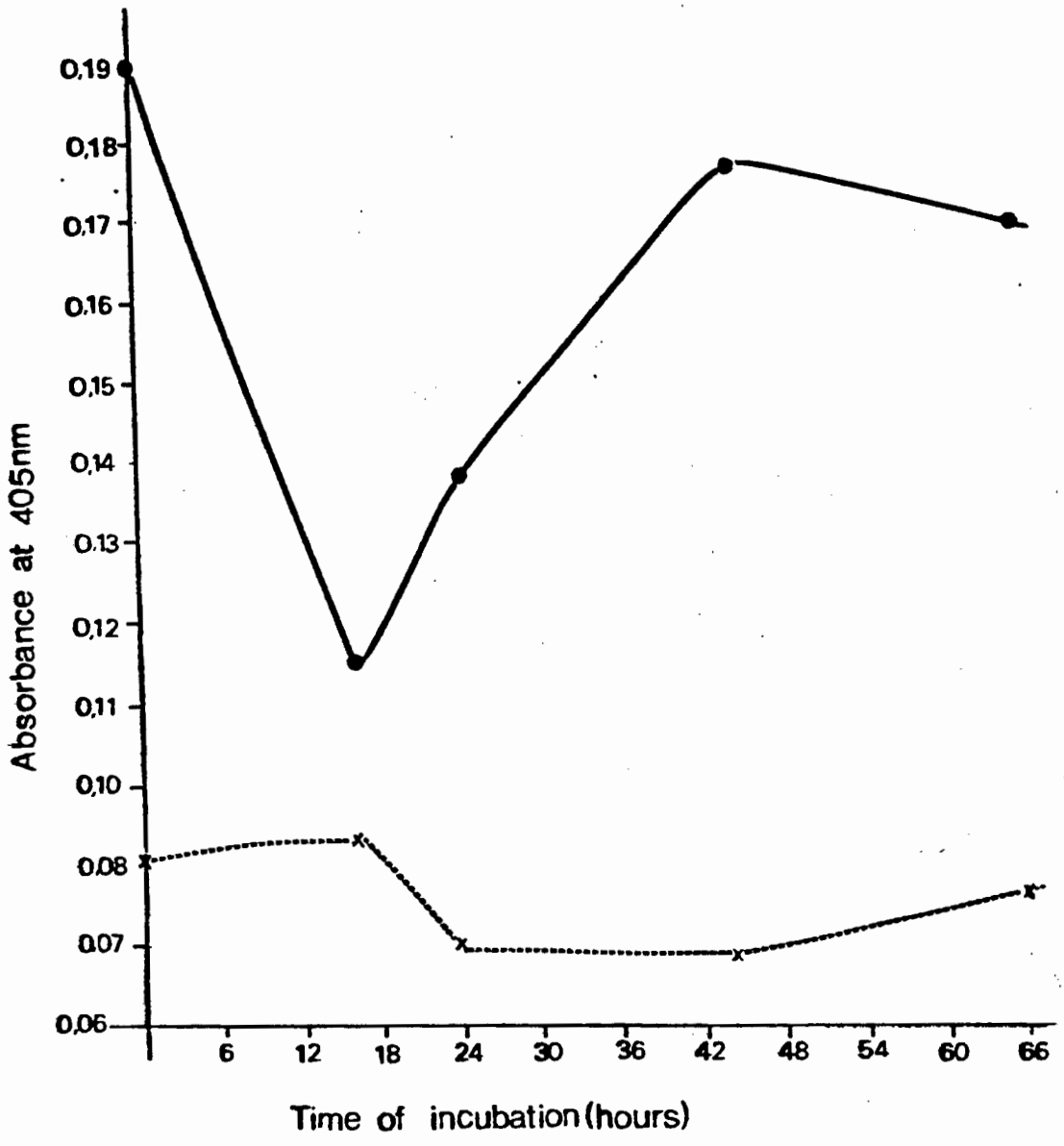


Figure VI.8: Graph to show infection curve of maize protoplasts infected with 2 ug/ml MDMV-B-ST. Protoplasts were inoculated in the presence of 2 ug/ml PLO and washed once in 0,7 M mannitol containing 100 mM $CaCl_2$. Protoplasts were assayed for the presence of MDMV-B-ST by DAS-ElISA. Anti-MDMV IgG and conjugate were used at 1/300 dilutions.

(●—●) -MDMV infected protoplasts

(x----x) -Uninfected protoplasts.

E. SIMULTANEOUS INFECTION OF MAIZE PROTOPLASTS WITH MDMV-B-ST AND CMV-K

1. Introduction

There have been several reports of infection of protoplasts both with unrelated viruses and with different strains of the same virus. Otsuki and Takebe (1976) showed that neither synergism nor antagonism occurred when tobacco protoplasts were doubly infected with TMV and CMV. The rates of replication of the two viruses are affected however (see Chapter II). Barker and Harrison (1978) showed that interference between two strains of raspberry ringspot virus (RRV) occurred and increased with increasing interval between inoculation of one strain and that of the other. It was shown that protection was not complete when one strain was inoculated to protoplasts which had been isolated from leaves systemically infected with the other strain.

To investigate the interaction of MDMV-B-ST and CMV-K, maize protoplasts were infected with the two viruses simultaneously as described below.

2. Inoculation of MDMV-B-ST and CMV-K in combination

Maize protoplasts were isolated and infected with a mixture of MDMV-B-ST (2 ug/ml) and CMV-K (50 ug/ml) in the presence of 2 ug/ml PLO in 0,02 M potassium

phosphate (pH 5,6) containing 0,7 M mannitol (VI.D.3). Protoplast samples were assayed at intervals during a 66 hour incubation period by DAS-ELISA (Figure VI.9). Controls consisted of protoplasts infected with only one virus i.e. only CMV-K or only MDMV-B-ST.

3. Results

Infection curves were plotted using average readings of three experiments (Figure VI.9). There appeared to be no infection by MDMV-B-ST in the maize protoplasts. In contrast, CMV-K infection and replication occurred. The infection curves for CMV-K inoculated alone and for MDMV-B-ST/CMV-K inoculated simultaneously, are similar. Thus the presence of MDMV-B-ST in the inoculum had no effect on the infection with CMV-K . In contrast it appeared that CMV-K prevented MDMV-B-ST infection.

Possible explanations for this are the following:

a) The higher concentration of CMV-K in the inoculum (50 ug/ml). This would mean that more CMV-PLO complexes would be formed than MDMV-PLO complexes. CMV would have a greater chance of occupying infection sites on the plasma membrane of the maize protoplasts.

b) Interference; CMV-K interferes with the replication of MDMV-B-ST in the protoplasts (see Discussion).

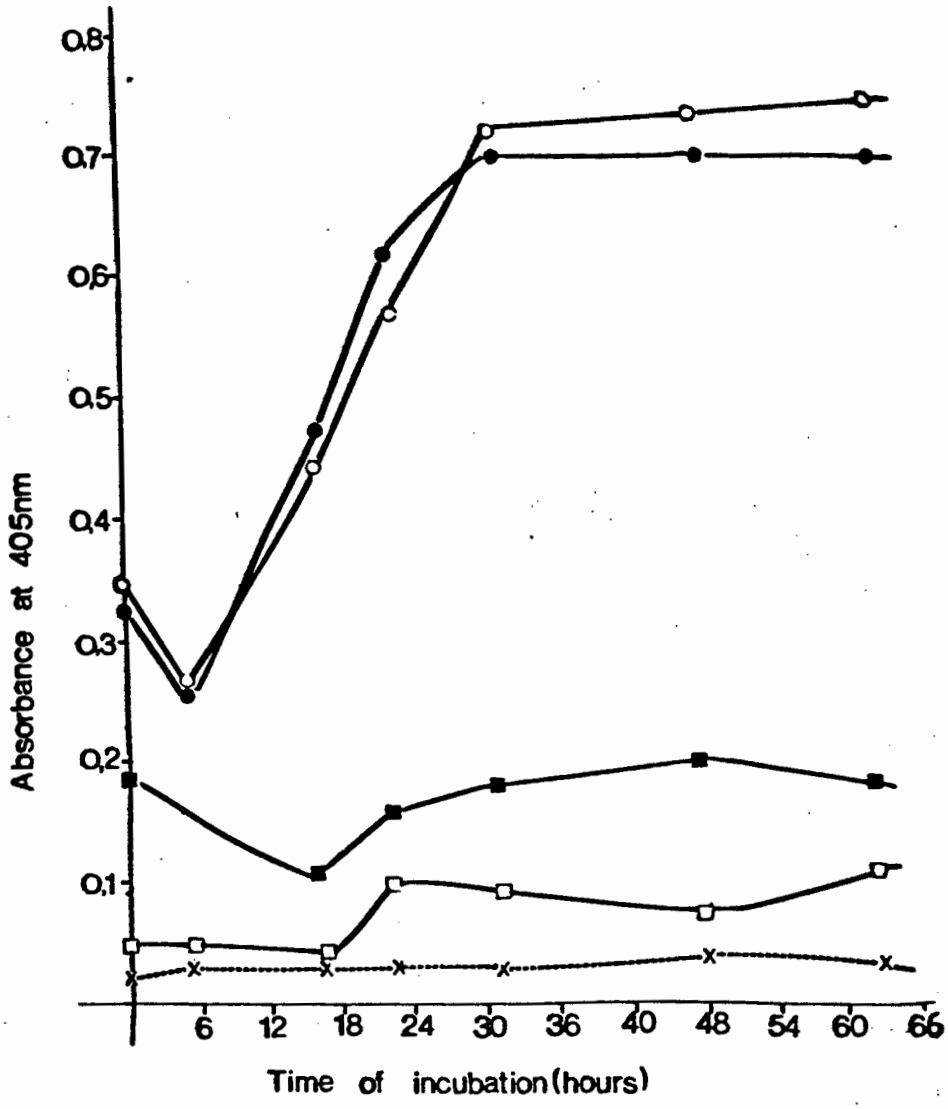


Figure VI.9: Graph to show infection curves for maize protoplasts inoculated with MDMV-B-ST (2 ug/ml) and CMV-K (50 ug/ml) simultaneously in the presence of 2 ug/ml PLO as described in VI.D.2. Uninfected protoplasts were inoculated with 50 ug/ml CMV-K alone (VI.C.2 and 3) and 2 ug/ml MDMV-B-ST alone (VI.D.2). Levels of CMV-K and MDMV-B-ST were assayed by DAS-ELISA using respective IgG's and conjugates at a 1/300 dilution.

(□—□) -Levels of MDMV-B-ST when protoplasts inoculated with CMV-K and MDMV-B-ST simultaneously.

(■—■) -Levels of MDMV-B-ST when protoplasts infected with MDMV-B-ST only.

(○—○) -Levels of CMV-K when protoplasts inoculated with MDMV-B-ST and CMV-K simultaneously.

(●—●) -Levels of CMV-K when protoplasts inoculated with CMV-K only.

(*····*) -Uninfected protoplasts.

F. ISOLATION OF PROTOPLASTS FROM SYSTEMICALLY INFECTED PLANTS (SAP-INOCULATED).

1. Isolation of protoplasts from MDMV-B-ST-infected maize seedlings

Protoplasts were isolated from 10 day old maize seedlings which had been sap-inoculated with MDMV-B-ST and had developed characteristic MDMV mosaic symptoms. The procedure used was the same as that described in VI.A. Following isolation the protoplasts were immediately washed, frozen, thawed, homogenized as described previously and the presence of MDMV-ST detected by DAS-ELISA (IX.D.5). The supernatant of the final wash of the protoplasts was kept to test for the presence of virus. The results are presented in Table VI.6.

It is evident from these results that protoplasts infected with MDMV-B-ST were isolated. It may be assumed that not every protoplast contained MDMV-B-ST, probably only a small percentage would be harbouring the virus.

Immuno-electroblotting confirmed the results obtained by DAS-ELISA. Protoplasts isolated from systemically infected seedlings were washed, the final

Table VI.6: Assay for the presence of MDMV-B-ST in protoplasts isolated from systemically infected maize-A seedlings.

Antigen[1]	Absorbance at 405 nm[3]
MDMV-B-ST sap[2] (positive control)	1,274
Uninfected protoplasts[4]	0,066
Protoplasts from MDMV-B-ST infected plants[5]	0,343
Supernatant after washing[6]	0,054

1. All antigens were diluted 1/2 in post-coating buffer (IX.A.3.(c)).
2. A leaf from the systemically infected seedling was crushed.
3. Samples were tested by DAS-ELISA. Anti-MDMV-B-ST IgG and conjugate were used at a 1/300 dilution.
4. Protoplasts were isolated from uninfected maize seedlings.
5. Immediately after isolation, protoplasts from systemically infected seedlings were tested for the presence of MDMV-B-ST.
6. The supernatant after washing the protoplasts from systemically infected seedlings was tested.

pellet resuspended in dissociation mix (IX.A.4.(e)) and subjected to PAGE gel electrophoresis (IX.D.6) and immuno-electroblotting (IX.D.7). The electroblot was probed with anti-MDMV-B-ST serum. Figure VI.10 indicates that the 37 kd protein of MDMV is present in the protoplast extract (lane 1). No virus particles were detected in the supernatant disrupted after washing the protoplasts.

2. Isolation of protoplasts from CMV-K-infected maize seedlings

Although maize protoplasts had been successfully infected with CMV-K (VI.C), it was of interest to investigate the feasibility of isolating protoplasts from seedlings, systemically infected with cucumber mosaic virus. A similar procedure to that carried out for isolation of protoplasts from maize seedlings systemically infected with MDMV-ST was used. Four day old maize-A seedlings were sap-inoculated with CMV-K which had been propagated on glutinosa tobacco. After 5-6 days protoplasts were isolated as described in VI.A. These were washed, homogenized as before and assayed for the presence of CMV by DAS-ELISA (IX.D.5). The results presented in Table VI.7 show that low concentrations of CMV were present in the maize protoplasts prepared from infected maize seedlings.

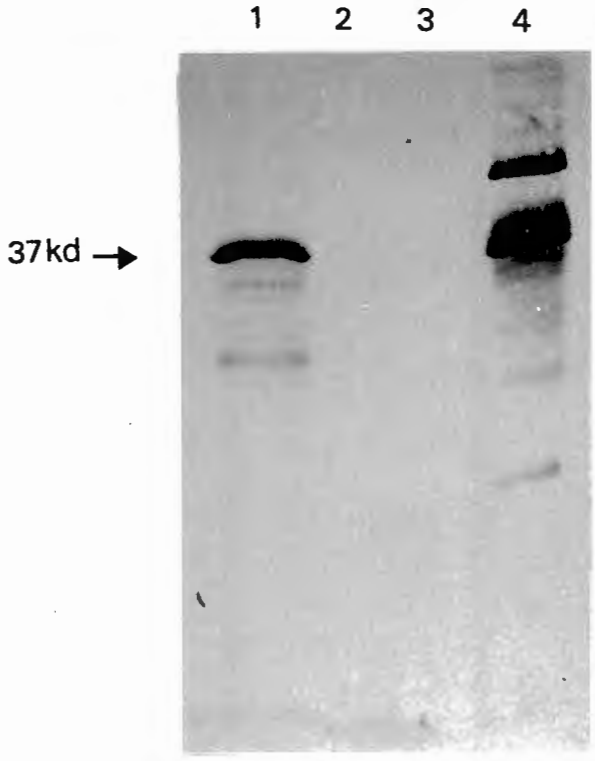


Figure VI.10: MDMV-B-ST infected protoplasts. Protoplasts were prepared from systemically infected plants and immuno-electroblotted against anti-MDMV-B-ST serum diluted 1/30. The 37 kd protein of MDMV-B-ST could be detected in the protoplast extract (lane 1).

Lane - 1 MDMV-B-ST infected protoplasts

- 2 Uninfected protoplasts

- 3 Supernatant after washing protoplasts

- 4 MDMV-B-ST standard.

Table VI.7: Assay for the presence of CMV-K in protoplasts isolated from systemically infected maize-A seedlings.

Antigen[1]	Absorbance at 405 nm[3]
CMV-K sap[2]	1,360
Uninfected protoplasts[4]	0,082
Protoplasts from systemically infected seedlings[5]	0,284
Supernatant after washing[6]	0,074

1. All antigens were diluted 1/2 in post-coating buffer (IX.A.3.(c)).
2. A leaf from the systemically infected seedlings was crushed.
3. Samples were tested by DAS-ELISA. Anti-CMV-K IgG and conjugate were used at a 1/300 dilution.
4. Protoplasts from uninfected maize seedlings.
5. After isolation protoplasts were tested for the presence of CMV-K.
6. The supernatant after washing the protoplasts was tested.

It must again be realized that not all protoplasts isolated would be infected with CMV-K and that some protoplasts may have received an incomplete complement of the genome.

3. Protoplasts isolated from doubly infected maize seedlings

It was previously shown that CMV and MDMV occur as natural double infections (Chapter III). Double infections could also be set up in the laboratory by sap-inoculation of plants with the two viruses (Chapter IV). Knowing that the two viruses occur in infected maize plants simultaneously, led to the next step which was to isolate protoplasts from double infected plants.

Plants that had been inoculated with a mixture of the two viruses were assayed by DAS-ELISA (IX.D.5) to identify those plants which contained both viruses. This step was essential as tests conducted on doubly infected plants showed that even if maize seedlings were inoculated with a mixture of two viruses, not all plants contained both viruses (see Results, V.D). Thus, protoplasts were isolated six days after inoculation (VI.A), washed, homogenized and tested for the presence of the two viruses by DAS-ELISA (Table VI.8).

Table VI.8: Assay for the presence of MDMV-B-ST and CMV-K in protoplasts isolated from doubly infected maize-A seedlings.

Antigen[1]	Absorbance at 405 nm[2]	
	Anti-MDMV-B-ST	Anti-CMV-K
MDMV-B-ST sap[3]	0,933	0,078
CMV-K sap[4]	0,068	1,442
Sap of doubly infected seedling[5]	0,642	0,312
Protoplasts from doubly infected[6] seedling	0,328	0,210
Uninfected protoplasts[7]	0,023	0,012

1. All antigens were diluted 1/2 in post-coating buffer (IX.A.3.(c)).
2. Samples were tested by DAS-ELISA. Anti-MDMV-B-ST and anti-CMV-K IgG and conjugate were used at a 1/300 dilution.

3/4. A leaf from maize cv.KEP infected with MDMV-B-ST and from glutinosa tobacco infected with CMV-K were tested (positive controls)

5. A leaf of maize-A which was doubly infected was tested.
6. Protoplasts from doubly infected seedling.
7. Protoplasts from uninfected maize-A.

The presence of both MDMV and CMV in the protoplasts could be detected. However this assay gives no indication of whether the individual protoplasts in the suspension are infected simultaneously with both viruses i.e. some protoplasts may be infected with only MDMV or only CMV; a small proportion may have both (see Chapter VIII, Discussion).

4. Infectivity of infected protoplasts

In order to assess whether the virus particles, in the protoplasts which had been prepared from infected seedlings, were infectious the following was done. Protoplasts were isolated fromm seedlings systemically infected with CMV-K, MDMV-B-ST or both. After incubation for 36 hours they were washed, frozen, thawed, homogenized and a little celite added. The homogenates were inoculated on to either 4 day old maize (cv. KEP) or squash plants at the dicotyledonous stage. The following was observed:

i) Protoplasts isolated from CMV-K-infected seedlings when inoculated on to squash caused characteristic distortion and crumpling of leaves (see Figure IV.1.(b)).

ii) Protoplasts isolated from MDMV-B-ST-infected seedlings produced a faint mosaic on inoculated maize.

iii) The protoplasts prepared from doubly infected seedlings were inoculated on to both squash and maize. Both hosts produced symptoms characteristic of CMV and MDMV respectively.

Thus even when the protoplasts had been incubated for some time the virus particles within them remained infectious.

G. INFECTION OF PROTOPLASTS FROM SYSTEMICALLY INFECTED PLANTS

1. Inoculation of protoplasts prepared from MDMV-B-ST-infected seedlings with CMV-K.

Synergistic and antagonistic interactions in protoplasts infected with two unrelated viruses or strains of the same virus have been reported (Barker and Harrison, 1976, 1978; Otsuki and Takebe, 1976, 1978; Watts and Dawson, 1980) (see Chapter II, Literature Review).

To investigate if the presence of MDMV had an effect on the infection with CMV-K and its replication the following was done:

Protoplasts were isolated from maize-A seedlings which had been sap-inoculated with MDMV-B-ST (VI.D.1). They were subsequently infected with CMV-K as described in VI.C.2. After varying time intervals one millilitre samples of approximately 1 x 10^5 protoplasts/ml were washed, frozen, thawed and homogenized, and assayed for the presence of the two viruses by DAS-ELISA (IX.D.5). In a parallel test, protoplasts from uninfected seedlings were isolated and inoculated with CMV-K (VI.C.2). Samples of these protoplasts were assayed concurrently. The results are presented in Figure VI.11. From the graph it can be seen that the presence of MDMV

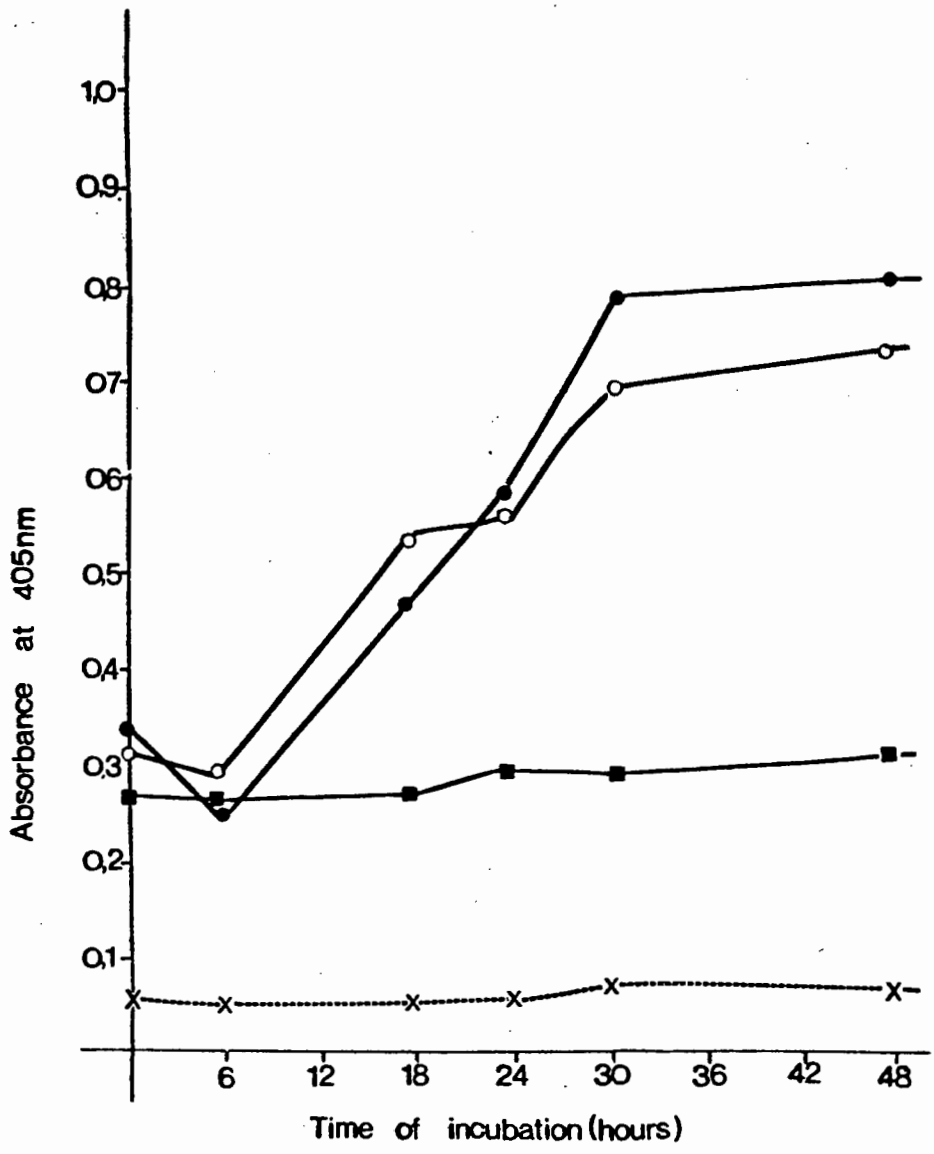


Figure VI.11: Graph to show infection of protoplasts isolated from MDMV-B-ST infected maize seedlings with CMV-K (50 ug/ml) as described in VI.C.2. The presence of the two viruses was assayed during a 48 hour incubation period. Readings are the average of two independent experiments. Anti-MDMV-B-ST and anti-CMV-K were used at a 1/300 dilution.

(●——●) -levels of CMV-K in protoplasts from uninfected seedlings inoculated with CMV-K alone.

(○——○) -levels of CMV-K in protoplasts from MDMV-B-ST infected seedlings inoculated with CMV-K.

(■——■) -levels of MDMV-B-ST in protoplasts from leaves of MDMV-B-ST infected seedlings

(×----×) -Protoplasts from uninfected plants.

in some of the protoplasts had little effect on their subsequent infection with CMV-K. It is clear that the infection curves for CMV in protoplasts from both uninfected and MDMV-infected seedlings follow similar lines. The amount of MDMV present in the protoplasts isolated from infected seedlings remained the same during the time of incubation; there was little replication and only a slight increase in the number of MDMV particles (see Figures VI.8.a, VI.9).

2. Inoculation of protoplasts prepared from CMV-K-infected seedlings with MDMV-B-ST

To investigate further the effect of these two viruses in protoplasts, maize protoplasts were isolated from seedlings which had been sap-inoculated with CMV-K. These were inoculated with MDMV-B-ST as described in VI.D.2. The infected and uninfected protoplasts (controls) were sampled at intervals during a 65 hour incubation period. In parallel, protoplasts were isolated from uninfected maize seedlings and inoculated with MDMV-B-ST.

Figure VI.12 shows the results obtained by DAS-ELISA (IX.D.5). Average readings from two separate experiments were recorded. It is interesting to note that there was very little, if any, multiplication of MDMV-B-ST when CMV was already present. It must be noted that not all the cells isolated from systemically infected plants would be infected with CMV. The low levels of MDMV-B-ST could be due therefore to those virus particles which had infected cells in which there was no CMV. This aspect was not pursued further, although FITC-labelled antibodies could possibly have differentiated them. The latter possibility was not fully explored (see Chapter VIII, Discussion).

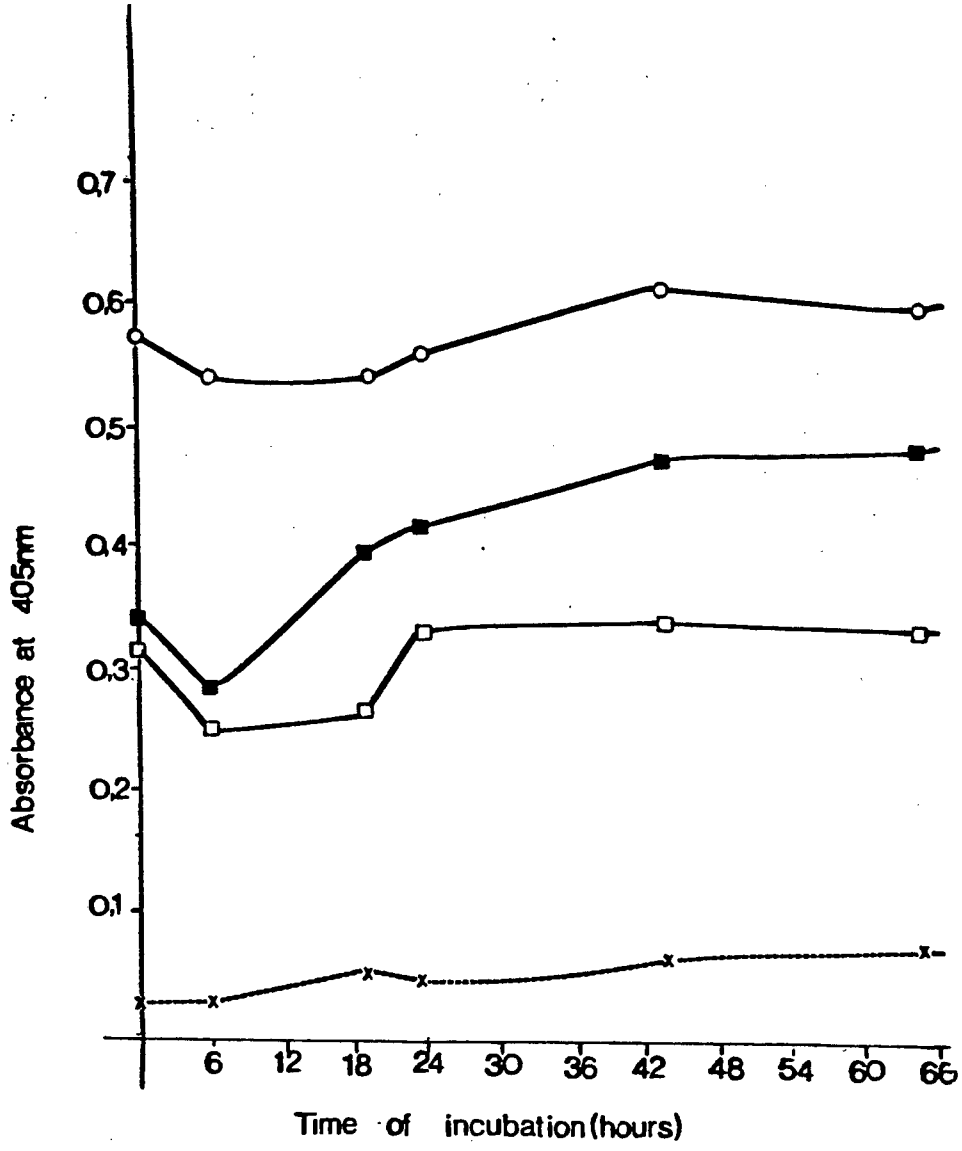


Figure VI.12: Graph to show infection of protoplasts isolated from CMV-K infected seedlings with MDMV-B-ST (2 ug/ml) as described in VI.D.2. The presence of the two viruses was assayed at intervals during a 66 hour incubation period by DAS-ELISA. Readings are averages of two independent experiments. Anti-MDMV-B-ST and anti-CMV-K IgG and conjugate were both used at a 1/300 dilution.

(o—o) -Levels of CMV-K in protoplasts isolated from seedlings systemically infected with CMV-K

(■—■) -Levels of MDMV-B-ST when inoculated into uninfected seedlings.

(□—□) -Levels of MDMV-B-ST when inoculated into protoplasts from CMV-infected seedlings.

(×—×) -Protoplasts from uninfected plants.

H. SURVIVAL OF PROTOPLASTS

To determine whether the presence of virus particles in protoplasts isolated from leaves of seedlings systemically infected with either MDMV-B-ST, CMV-K or both, affected their survival the following was done. Protoplasts were isolated from infected seedlings: (i) CMV-K infected only, (ii) MDMV-B-ST infected only or (iii) MDMV-B-ST/CMV-K infected as described previously (VI.A.2 and 3). The protoplasts were incubated as before (VI.A.3.c) and at various times samples were stained with Evans blue (IX.D.12). Evans blue, an exclusion dye, is commonly used to test the viability of cells. This dye will not pass through viable membranes. The number of surviving, viable protoplasts compared with the number of intact protoplasts in the suspension, was recorded. Figure VI.13 indicates that there was a slight reduction in the survival of the protoplasts from systemically infected leaves. However no significant difference between protoplast survival from singly and doubly infected leaves was evident.

MDMV and CMV have been shown to co-exist in intact plants so it is not surprising that they may both be present in a population of protoplasts without seriously affecting the survival of such cells. It must be noted that in the protoplasts from doubly infected leaves, it is not known what proportion of the protoplasts contain both MDMV and CMV simultaneously.

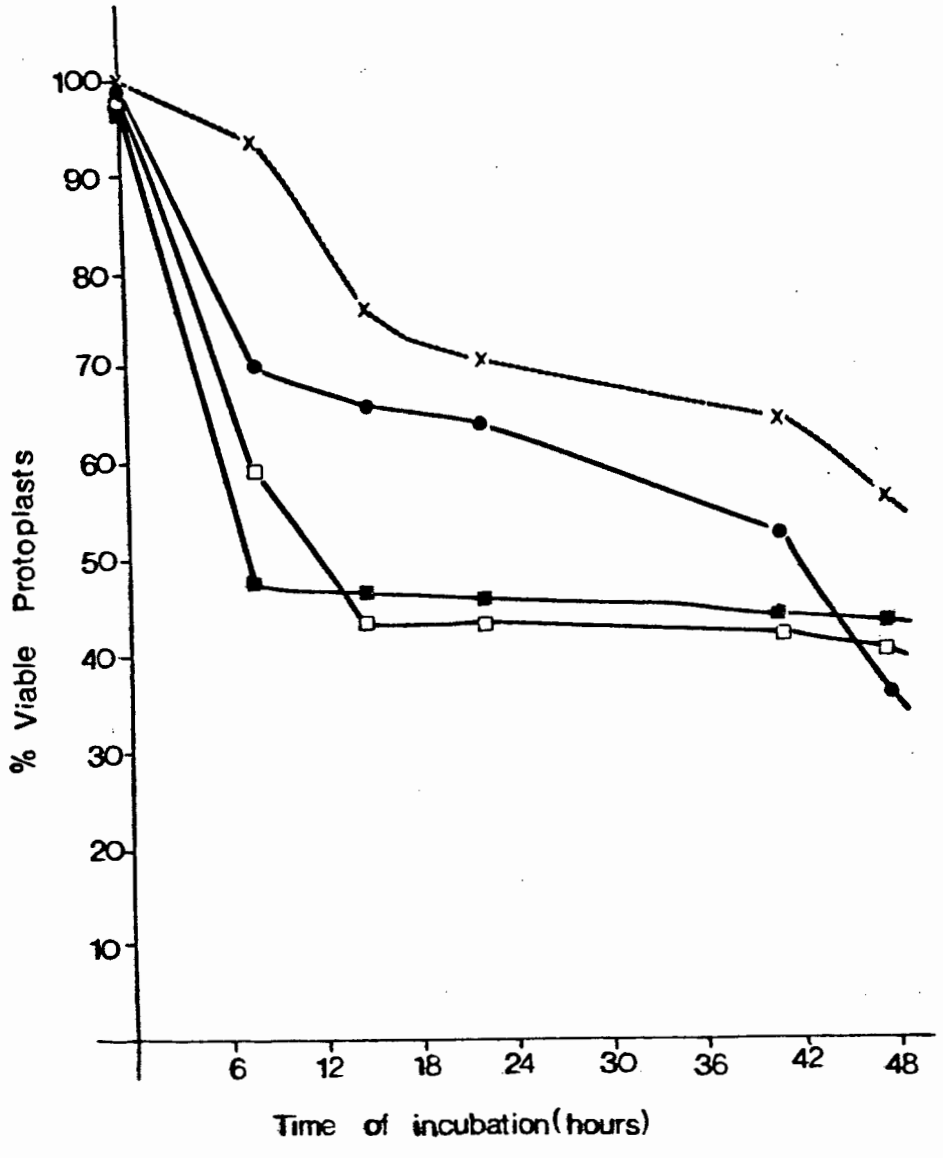


Figure VI.13: Survival of protoplasts isolated from systemically infected seedlings. Viability was assessed using Evans Blue and the average of three separate counts was used to estimate the final number of surviving protoplasts.

(x-----x) -Protoplasts from uninfected plants

(●——●) -Protoplasts from plants infected with CMV-K

(■——■) -Protoplasts from plants infected with MDMV-B-ST

(□——□) -Protoplasts from plants infected with both MDMV-B-ST and CMV-K.

I. CONCLUDING COMMENT

Experiments conducted showed that maize protoplasts could be isolated and maintained for at least 66 hours. It was possible to infect maize protoplasts with CMV-K and MDMV-B-ST. Double inoculation with MDMV-B-ST and CMV-K simultaneously, showed that the presence of MDMV-B-ST in the inoculum had no effect on the infection of the protoplasts by CMV-K. However, in the presence of CMV-K there was no infection by MDMV-B-ST in the maize protoplasts. Protoplasts were isolated from seedlings which were systemically infected with MDMV-B-ST, CMV-K or both. Protoplasts prepared from these plants could be super-infected with either MDMV-B-ST or CMV-K. It was observed that, when CMV-K was already present in some of the protoplasts subsequent infection by MDMV-B-ST was not possible. Protoplasts prepared from maize seedlings systemically infected with MDMV-B-ST could be super-infected with CMV-K. These results correlated to the findings with whole plants (see Chapter V). Evidence for doubly infected individual protoplasts within the population was not obtained.

CHAPTER VII

LIPOSOMES

A. INTRODUCTION

Liposomes are lipid vesicles which have often been used for delivery of various compounds and particles to both animal and plant cells (Tyrell et al., 1976; Fukunaga et al., 1981). Liposomes may be formed by several methods using different types of phospholipids (see Chapter II).

Liposome-mediated delivery of both virus particles and their RNA often enhances the efficiency of infection. The instability of CMV and the difficulty associated with MDMV infection of protoplasts made the liposome system attractive. Liposomes protect the RNA, which they encapsulate, from degrading by RNases. Thus the possibility of delivery of CMV and MDMV (and their RNAs) into protoplasts by liposomes was investigated.

B. PRODUCTION OF LIPOSOMES

The first step in the investigation was to optimise methods for liposome production. The method of Szoka et al. (1978) was attempted.

Four differently charged liposomes could be made depending on the type of phospholipid used:

a) Phosphotidylcholine (PC) liposomes

b) Phosphotidylserine (PS) liposomes

c) Phosphotidylcholine: cholesterol (PC:chol) liposomes

d) Phosphotidylserine: cholesterol (PS:chol) liposomes

For PC and PS liposomes, 10 uM phosphotidylcholine or 5 uM phosphotidylserine (Sigma Chemicals) were dissolved in 10 ml chloroform (Merck Analar) in a round bottomed flask. For PC:chol and PS:chol liposomes, 5 uM cholesterol (M_r = 350) in chloroform was added to the PC- or PS-chloroform mixture. The choloroform was evaporated under a vacumn using a rotary evaporator (Rotavapor-EL, Büchl) for approximately one hour. The monolayer of lipid which had formed on the bottom of the flask was redissolved in 0,5 ml ether, 0,12 ml liposome buffer (IX.A.8.(a)) and 0,1 ml PBS buffer (IX.A.1.(b)). The latter constituted the aqueous phase. The mixture was mixed for 20 seconds on a Vortex mixer, flushed with nitrogen gas for a few seconds and then sonicated (Soniprep 150,MSE) for three bursts of 5 seconds each at full power (28 minutes amplitude). The remaining ether was rotary evaporated for approximately half an hour. The liposomes thus prepared were microscopically examined using a Zeiss microscope (1M35).

Figure VII.1.(a) shows that various sizes of liposomes resulted. The longer the preparation was sonicated the greater the number of small unilamellar vesicles in the liposome suspension. No difference could be distinguished between PC, PS, PC:chol and PS:chol liposomes microscopically.

C. INCORPORATION OF FLUORESCENT DYES

To investigate the conditions required for liposomes to encapsulate compounds, two fluorescent dyes were chosen since their encapsulation could be easily visualized by fluorescent microscopy.

1. Fluorescein diacetate (FDA).

Cassells (1978) incorporated FDA into liposomes and fused these to tomato protoplasts. A procedure similar to that of Cassells' was followed in the present investigation.

A 5 mg/ml solution of FDA (Sigma Chemicals) was prepared in acetone. Liposomes were prepared as described in VII.B except that 0.01% (v/v) fluorescein diacetate and 150 ul HEPES buffer (IX.A.8.(b)) constituted the aqueous phase instead of PBS buffer. Liposomes were washed after preparation by three cycles of centrifugation for four minutes in an Eppendorf centrifuge (Model 5413) using liposome buffer (IX.A.8.(a)) to remove free FDA.

2. Calcein

A solution of calcein 2', 7'[(bis[carboxymethyl]-amino)methyl] fluorescein] (Sigma; M_r = 675) was prepared by M. Friede, Department of Biochemistry, UCT. Liposomes were prepared as in VII.B. Fifty microlitres calcein and 150 ul HEPES buffer (IX.A.8.(b)) constituted the aqueous phase. To separate unencapsulated dye, 0,5 ml of the liposome preparation were mixed with 3 ml of 30% w/v Ficoll solution (Sigma Chemicals, M_r = 400 000) in liposome buffer in a Beckman 50,1 SW centrifuge tube. Three mililitres of a 10% (w/v) Ficoll solution in the same buffer and 1 ml of liposome buffer were sequentially layered over the sample. The gradient thus formed was centrifuged at 30 000 rpm for 30 minutes. The liposome band could be collected from the liposome/10% ficoll interface. The liposomes were viewed using a Zeiss IM35 microscope fitted with filters etc for fluorescent microscopy (VI.C.4). Figure VII.2.a shows small unilamellar vesicles containing calcein.

D. FUSION OF LIPOSOMES TO MAIZE PROTOPLASTS

1. Liposomes containing fluorescent dyes

Fusion of liposomes containing fluorescent dye to protoplasts facilitates visualization of the process since the fluorescent dye will cause protoplasts to fluoresce. The method used was modified from that of Szoka et al. (1978) (M. Friede, Dept. of Biochemistry, U.C.T., personal communication) and is outlined below.

a) FDA-containing liposomes were prepared (VII.C.1 except that 0,7 M mannitol was dissolved in HEPES buffer before it was added to the lipid monolayer. A further 0,5 ml of HEPES/0,7 M mannitol was added to the liposome suspension after their preparation. The unincorporated FDA was removed by gradient centrifugation (VII.C.2). Maize protoplasts were isolated (VI.A.2 and 3), 1 x 10^6 protoplasts/ml pelleted by centrifugation and gently resuspended in one millilitre of 0,7 M mannitol. To this 0,5 ml of the liposome preparation and 0,5 ml of HEPES/0,7M mannitol were added. The protoplast-liposome mixture was left for 10-20 minutes at 22°C. Two hundred and fifty microlitres of 0,7 M mannitol containing 20% (w/v) PEG

(M_r = 6 000) was added. The mixture was left for a further 5 minutes. The protoplasts were washed by three cycles of centrifugation and resuspended in liposome buffer containing 0,7 M mannitol before viewing.

b) Calcein-containing liposomes

To investigate which type of liposome was most suitable for fusion with maize protoplasts, different phospholipid combinations were used to make liposomes with incorporated calcein. PC, PS:chol and PC:chol liposomes were made as described in VI.B and C.2.

Maize protoplasts were pelleted as before and resuspended in 1 ml of 0,7 M mannitol and 0,5 ml of the liposome preparation. Thirty seconds later 0,5 ml of 20% (w/v) PEG (M_r = 6000) in 0,7 M mannitol was added. The viscous liposome protoplasts suspension was left for 10 minutes before the viscosity was reduced by the addition of 20 ml of fusion medium (IX.A.8.(c)). After a further ten minutes, the protoplasts were washed as before using 0,7 M mannitol. The protoplasts were viewed microscopically at various times after the fusion procedure.

c) Results

No fusion of liposomes containing FDA or calcein occurred in the absence of HEPES buffer. FDA is commonly used to assay the viability of the plasmalemma by its exclusion by viable membranes (Larkin, 1976). It is essential to remove all unencapsulated FDA from the liposome preparation before fusion with the protoplasts.

There are two advantages in the use of calcein for incorporation into liposomes and their fusion to protoplasts:

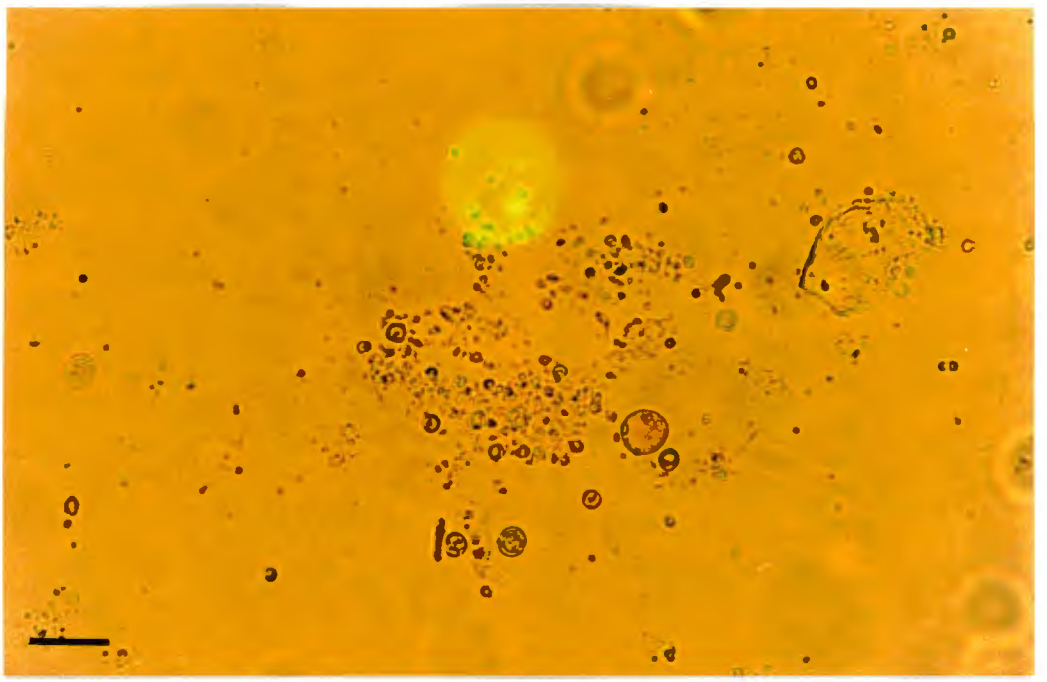
i) Calcein will only traverse the protoplast membrane if fusion with the liposomes has occurred. No passive diffusion of the dye can occur.

ii) The intensity of the fluorescence with calcein increases on dilution so that protoplasts which have fused to the calcein-containing liposomes fluoresce more brightly than the liposomes themselves.

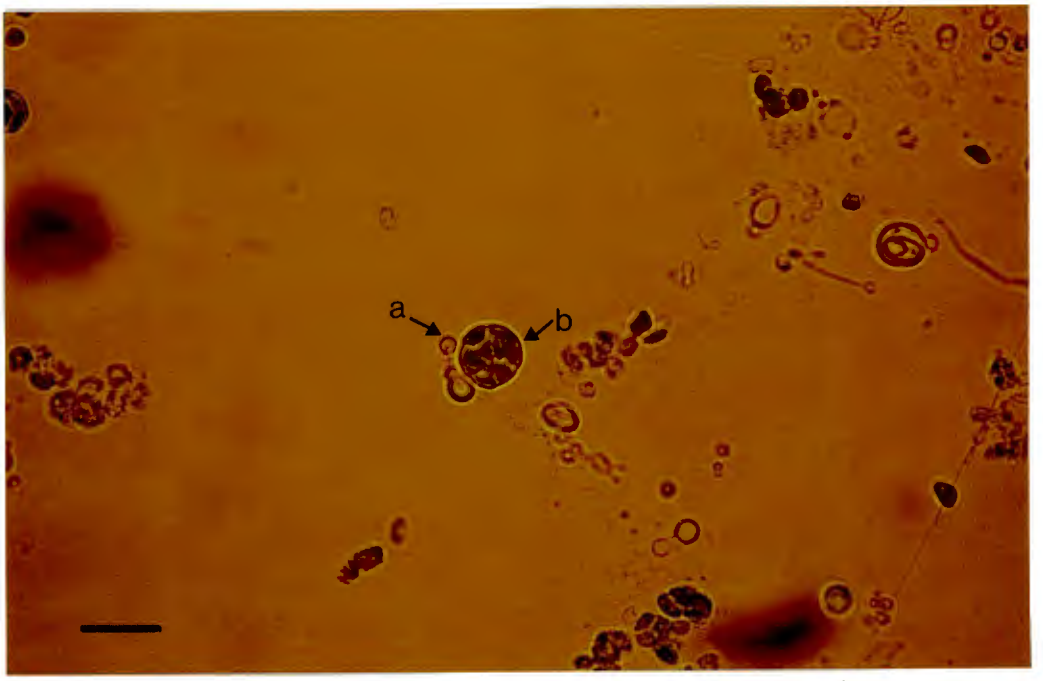
PEG was essential for fusion to occur; no fusion occurred in its absence. The concentration of PEG in the fusion medium was important; at concentrations greater than 20% (w/v), the protoplasts were destroyed. Figure VII.1.b shows fusion of liposomes to a maize protoplast.

Figure VII.2.a, b, c and d show fusion of liposomes containing calcein to maize protoplasts.

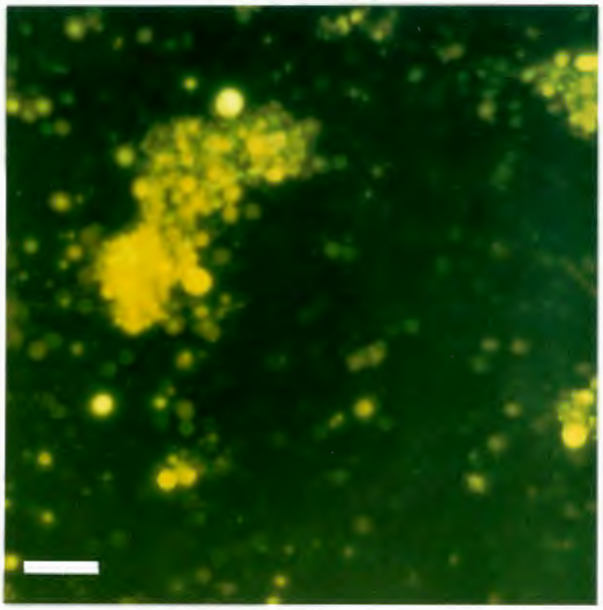
Figure VII.1: Liposomes and their fusion to maize protoplasts.



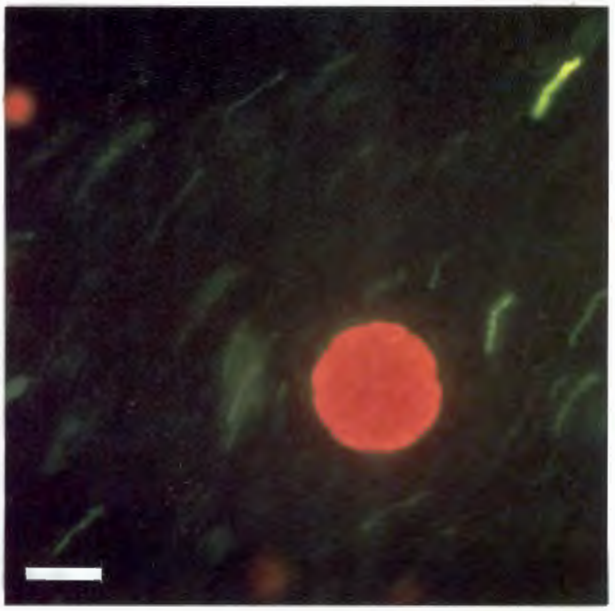
a) Population of small unilamellar vesicles and large multimellar vesicles prepared by reverse phase evaporation and viewed by light microscopy. Magnification was 280X. Bar represents 25 um.



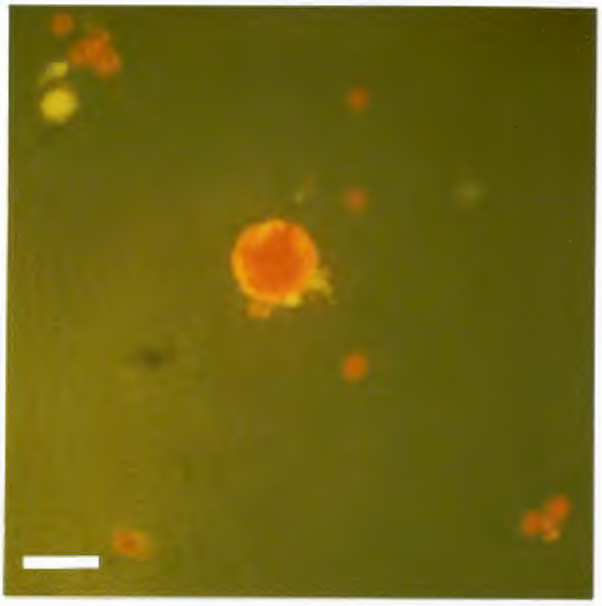
b) Fusion of a maize protoplast (a) and small liposomes (b). Magnification was 280X. Bar represents 25 um.



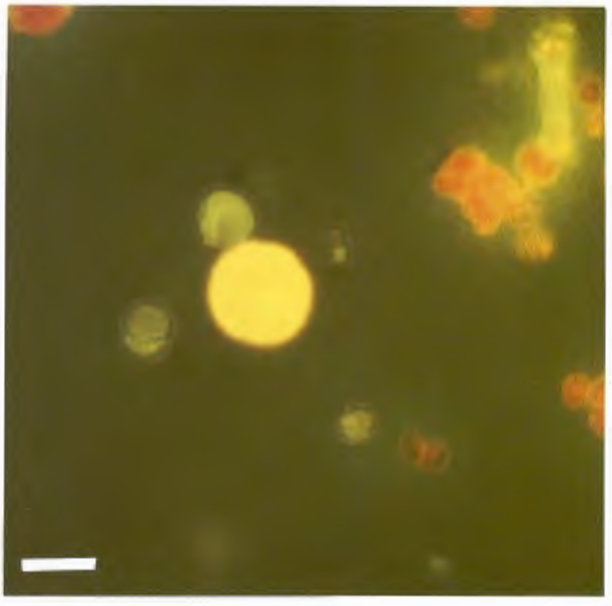
a



b



c



d

Figure VII.2: Fusion of liposomes containing calcein to maize protoplasts. In all photographs the magnification was 280x and the bar represents 15 um.

a) Population of liposomes, heterogenous in size, prepared by reverse phase evaporation.

b) Maize protoplast viewed using fluorescent microscopy and showing red autofluorescence.

c) Liposome containing calcein adsorbed to the protoplast membrane.

d) Liposome has fused with the protoplast releasing calcein into it so that it now fluoresces yellow.

2. Encapsulation of virus in liposomes

Fusion of liposomes containing fluorescent dyes was successful and so the next step was to investigate the possibility of encapsulating virus particles (CMV-K or MDMV-B-ST) into the vesicles. Liposomes containing either CMV or MDMV could then be fused to maize protoplasts.

To encapsulate virus particles in liposomes some modifications to liposome production had to be considered. Liposome preparation includes a step of sonication (VII.B) which could be destructive to viruses. When the instability of CMV is taken into account, this is particularly important. In addition the charge carried by the liposomes would influence their ability to incorporate the charged virus particles.

a) Freeze-dried liposomes

"Freeze-dried" liposomes made by the dehydration-rehydration method of Kirby and Gregoriadis (1984) were produced since the steps involved in their preparation are conducive to maintaining the virus particles in an intact infectious state. The method is described below.

Sixty-six microlitres of a 100 mg/ml PC stock solution were dissolved in 5 ml of chloroform in a round bottomed flask. The chloroform was evaporated under vacuum as described before, flushed with nitrogen gas and 4 ml of sterile distilled water added. The suspension was 'vortexed' for 2-3 minutes and sonicated for 3 minutes. The small unilamellar vesicles which resulted, were flash frozen in liquid nitrogen and freeze-dried overnight. Purified CMV-K (6 mg/ml) was diluted 1/10 in 0,005 M borate buffer (IX.A.2.(b)) and 2 ml added to the freeze-dried liposome preparation. The virus-liposome mixture was gently swirled for 10 minutes by circular wrist movement.

b) Multilamellar vesicles (MLV's)

Since no sonication is involved in the production of MLVs, they too appeared to be ideal for encapsulation. The method for their preparation is summarised below: (M. Friede, Dept. of Biochemistry, U.C.T., personal communication).

To a monolayer of phospholipid (PC or PS) in a round bottomed flask, 1 ml of a 1/10 dilution of a 6 mg/ml preparation of CMV-K (in 0,005 M borate buffer) was added. The round bottomed flask was gently swirled for ten minutes.

c) Reverse phase evaporation vesicles (REV's)

The method of Szoka et al. (1978) for preparing REV's was modified to exclude the sonication step but was similar to that described in VII.B. To a monolayer of PC prepared as described previously, 0,3 ml of a 0,6 mg/ml CMV-K suspension (1/10 dilution of 6 mg/ml CMV-K preparation) and 1 ml of ether were added. The ether was evaporated for one hour under reduced pressure (not measured) 0,3 ml of PBS buffer added and the resulting liposome-virus preparation passed ten times through a syringe fitted with a needle (28 gauge). This was done to simulate the action of sonication.

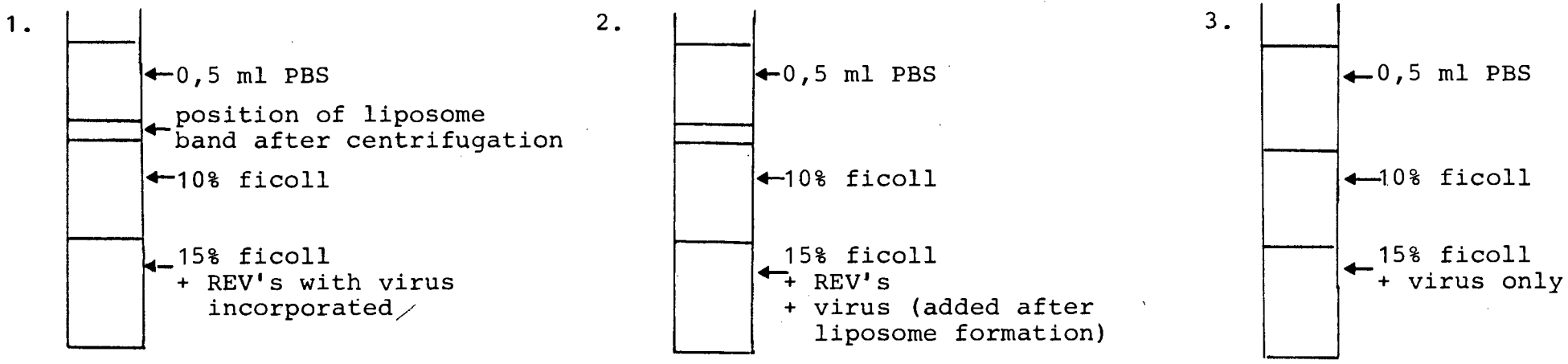
d) Incorporation of radiolabelled virus into liposomes

Radiolabelling virus particles facilitates their detection after their encapsulation into liposomes. CMV-K was labelled using ^{35}S protein labelling reagent (^{35}SLR, Amersham) (see IX.D.10) and incorporated into REV's as described in VII.D.2.(c).

e) Separation of unencapsulated virus

Free virus particles i.e. those which had not been incorporated into the liposomes or had merely attached to the liposome surface were separated by gradient centrifugation as follows. One and a half millilitres of 15% (w/v) Ficoll (M_r = 400 000) in PBS buffer was added to a Wasserman tube, 0,3 ml of the liposome:virus preparation (either freeze-dried, REV's or MLV's) were added followed by 0,5 ml PBS buffer. The gradients were centrifuged for 5-6 minutes on a bench centrifuge (BGH type) at full speed (approximately 3 000 rpm). The liposomes floated to the interface of the 10% ficoll solution and the PBS buffer (Figure VII.3). The layers of the gradient were separated and transferred to Eppendorf microtubes. They were stored at 4°C until required for testing by DAS-ELISA (IX.D.5).

Figure VII.3: Diagram to show layers formed by gradient centrifugation.



f) Controls

For each type of liposome preparation the following controls were included (see Figure VII.3).

i) Liposomes prepared without the incorporation of virus. Instead of CMV-K, PBS buffer was added. To these liposomes, encapsulating PBS buffer only, 0,3 ml of 0,6 mg/ml CMV-K was added before separation by gradient centrifugation to ensure that this separation step was removing free CMV-K efficiently.

ii) Only virus was added to the 15% ficoll layer i.e. no liposomes.

iii) Liposomes were prepared without the incorporation of CMV-K and were layered on the 15% ficoll layer.

g) Assay for the presence of virus in the liposomes

To test for the incorporation of CMV-K in the liposomes, the layers of each gradient were i) diluted four-fold in PBS buffer containing 0,1% (v/v) Triton X-100 and 0,2% BSA. (Triton X-100 as detergent, causes the liposomes to burst and release their contents.) The samples were then tested by DAS-ELISA (IX.D.5) ii) liposomes containing labelled CMV-K were subjected to polyacrylamide gel electrophoresis and autoradiography (IX.D.11).

h) Results

From Table VII.1 it is evident that CMV particles must adsorb to the liposome surface since a high reading was obtained for liposomes which had had virus added after their formation (Fraction 2a). The gradient appeared to be fairly efficient at separating the free virus particles as fraction 3a gave only a background reading. The fraction containing liposomes with incorporated virus gave a reading only marginally higher than that for Fraction 2a. REV's, MLV's and freeze-dried liposomes gave similar results i.e. not convincing incorporation of CMV-K.

When the presence of labelled CMV-K in the liposomes was tested by autoradiography no CMV protein could be detected.

These results could be attributed to

i) the instability of the virus (see Figure IV.2 b and c)

ii) the method of preparing the liposomes was incorrect. The charge carried by the virus particles may have been such that few intact particles could be encapsulated in the liposomes (see Chapter VIII, Discussion).

Table VII.1. Assay for incorporation of CMV-K into reverse phase evaporation vesicles.

Fraction[1]		Absorbance at 405 nm[2]
1.a.	Liposomes with virus incorporated[3]	0,616
b.	10% ficoll layer	0,482
c.	15% ficoll layer	0,440
2.a.	Liposomes with virus added[4].	0,502
b.	10% ficoll layer	0,642
c.	15% ficoll layer	0,402
3.a.	Virus only[5]	0,133
b.	10% ficoll	0,570
c.	15% ficoll	0,807
CMV-K control[6]		0,768

1. The fractions after gradient centrifugation were diluted four-fold in PBS containing 0,1% v/v Triton X-100 and 0,2% BSA. The reading for the 1/16 dilution is given.
2. DAS-ELISA was used to assay for the presence of CMV-K. Anti-CMV-K IgG and conjugate were used at a 1/300 dilution.
3. REV's with CMV-K incorporated during their preparation.
4. REV's were prepared and then CMV-K added before centrifugation.
5. Only CMV-K was layered onto the gradient.
6. Positive control.

E. CONCLUDING COMMENT

Although incorporation of the fluorescent dyes, calcein and FDA, and their fusion to maize protoplasts was successful, it was considered that encapsulation of CMV-K was not sufficiently efficient to continue at that time with attempts to fuse the liposomes to the protoplasts.

CHAPTER VIII

DISCUSSION AND CONCLUSION

A. NATURAL FIELD INFECTIONS

In Chapter III results were presented of analyses done on infected field collected maize. Three of these i.e. 'McArthur', 'Roodeplaat B' and 'Hentie' maize showed a mixed infection of MDMV-B and CMV. Although these plants were the only ones that were investigated in some detail, many other similar examples were collected showing that similar mixed infections occurred more frequently (von Wechmar, unpublished).

As MDMV-B can occur as a seedborne virus (Shepherd and Holdeman, 1965; von Wechmar and Chauhan, 1984) and is also easily aphid transmitted in a non-persistent manner by several aphid species, it is likely that these two transmission mechanisms are activated in natural epidemiological situations. This assumption is not unrealistic if one considers that at least one seed source was found to have 1-3% seeds contaminated with MDMV. If seed contamination with MDMV-B is such that a high proportion of seeds will still germinate to give rise to MDMV-B-infected seedlings, the latter would be the primary source of infection from which secondary spread could take place. From this primary seed infection the aphids will thus disseminate the

virus to surrounding plants. If similar foci of infection occur throughout a large field, (as would be the case where MDMV is seedborne) these foci would soon enlarge and in a season which favours aphids, particularly severe aphid infestation may result in a 100% infection by the time the maize reaches maturity.

It is seldom that MDMV infection of maize will occur alone (Chapter III). We must now consider the source of the CMV which occurs in the mixed infection with MDMV. For the purposes of this discussion, known situations of CMV epidemiology will be taken into consideration (ie. Tomlinson, 1975; von Wechmar, pers. comm).

CMV is spread by the seeds of many weeds (see Chapter II, Literature Review). This implies that weeds may serve as reservoir hosts for the virus. One such example is Commelina benghalensis. This weed commonly occurs in South African maize fields. Two samples of C. benghalensis collected from maize fields were found to be infected with CMV (von Wechmar, unpublished results). The life cycle of this weed is such that it is difficult to eradicate (Grabrant, 1985). Other weeds commonly found growing in maize fields are listed in Chapter II. Many of these are CMV hosts and may be reservoirs of the virus (Tomlinson, 1970). Many aphid species transmit different strains of CMV in a non-persistent manner (Francki et al., 1979). In other weeds, aphid/virus and virus/host specificity is low. This leads to a situation where aphids may easily carry CMV from

crops such as tomatoes, green pepper, soyabeans, peanuts and lupins into maize fields or infect weeds thus starting a cycle of infection (Lupuwana, 1985; von Wechmar, unpublished).

The fact that many of the aphid species may have some host specificity or preference does not preclude them from probing maize or weed leaves and in this preliminary feeding action transmit the virus; short acquisition feedings lead to efficient transmission of CMV. In Chapter V it was shown that 1-3 minute acquisition feedings led to 13% CMV transmission with R. maidis and R. padi. Neither of these aphids normally colonize squash plants, but it was shown that under experimental conditions short probes on CMV-infected squash leaves followed by inoculation feeding on maize and squash plants led to a high percentage of virus transmission (see Table V.4).

How then do these two relatively similar infection cycles link up in maize plants grown under natural conditions? In the case of 'Hentie' maize (III.C.), the infected plants were collected during drought conditions. The green maize field was the most succulent vegetation in the whole environment. One could hypothesize that the aphids were experiencing stress conditions because of the drought, alatae forms would develop and migrate to the lush green vegetation i.e. the maize field. If the aphids had originated from CMV-infected source plants, this could be one way that the virus could be introduced into the maize.

Alternatively the infection cycle could have started earlier i.e. in the mother plant from which seed was collected. This seed could contain seedborne virus. Although evidence is available of CMV and MDMV in seed (Knox, 1983; von Wechmar and Chauhan, 1984) nothing is known about the mechanism and the time of host infection which would determine when and how the virus enters the embryos of the developing seed. It is not known whether it is the male or female gametes which introduce the virus. In barley stripe mosaic virus (BSMV) both male and female gametophytic cells have been shown to carry the virus and give rise to seedborne BSMV in barley (Carroll, 1974).

B. DETECTION OF MIXED INFECTIONS

Mixed infections often involve viruses which differ biochemically and biophysically. This point must be taken into consideration during extraction of the viruses from the doubly infected plant tissue.

MDMV and CMV are different in several respects i.e. stable/unstable; single genome/multipartite genome. Unless these facts are taken into account during the extraction process, it is almost certain that one or other component of the mixed infection will be eliminated before the final stage of purification is reached, with the result that only one virus will be detected.

For these reasons another approach was adopted (von Wechmar, unpublished). This is based on gentle extraction with emphasis on speed and concentration of viruses present in the infected tissue rather than clarification to remove all host and non-viral matter. The latter approach invariably eliminates a second virus if present. For extraction of MDMV and CMV from a mixed infection it is essential to use a buffer and pH that would stabilize the extremely unstable CMV particles (Figure IV.2.c). In addition, it would be essential to carry out the extraction in the shortest possible time (i.e. hours and not days). To retain the filamentous MDMV in the mixture a high pH and a relatively low speed during ultracentrifugation would be neccessary to

prevent aggregation, shearing and subsequent loss of the virus. These considerations were operational when investigating the 'Hentie'-, 'McArthur'- and 'Roodeplaat'-isolates (see Chapter III).

Immunization of rabbits with products extracted from the mixed natural infections raised antisera which was able to recognise both MDMV and CMV in single, purified preparations. IEB proved to be the best experimental method to evaluate such products (see Figure III.2.c and 8.b). However if antiserum of this nature is used in ELISA tests only, or in tests designed for filamentous (e.g. tube or microprecipitin tests) or iscosahedral viruses (e.g. Ouchterlony double diffusion test), interpretation of results may be incorrect. An example of such a problem was examined by von Wechmar et al. (1984) in their study of viruses infecting small grain crops. BMV was found to be seedborne, and caused conflicting results until it was discovered that seedborne virus could give rise to infected plants. Therefore supposedly uninfected control plants reacted positively in serological tests. This was initially attributed to non-specific background reactions. It was noted that in incidences where seedborne virus was detected serologically, the plants often had not exhibited symptoms.

C. MIXED INFECTIONS IN THE LABORATORY

Once it had been established that mixed infections occurred in the natural field situation, laboratory experiments were designed to explore double infections of MDMV and CMV in more detail.

1. Sap-inoculations of maize.

Sap-inoculation of the two viruses, MDMV and CMV, either by single virus inoculations, followed by a second virus after certain time intervals (see V.B) or by preparing inoculum containing both viruses and using that for inoculation, was carried out. It was hoped that plants infected in this manner would give an indication whether an inter-relationship existed which would be expressed by symptoms.

Table V.1 and 2 present the data obtained when individual plants were assayed for the presence of either CMV or MDMV. Table V.3 summarizes these results by quantifying the number of individual plants which contained both MDMV and CMV i.e. were doubly infected. It was evident that more plants contained both viruses together than either one or the other. It is clear therefore that MDMV and CMV can co-exist in the same plant. Since single plant analysis was done on all the sap-inoculated plants, it was possible to ascertain

the infection of each individual plant. No synergism or antagonism between the two viruses was evident. The decrease in the number of plants which were positive for CMV and MDMV when the presence of these two viruses was assessed separately compared to single infections, could be explained by the fact that the inoculum consisted of a 1:1 mixture of the two viruses (thus each was present in a 1/2 dilution) whereas for single infections the virus was undiluted when sap-inoculated. Plants were also inoculated with CMV-K only or MDMV-B-ST only. These were tested for both viruses, even though they had been inoculated with a single virus, so that seedborne virus, if present, could be detected. In these tests seed-transmitted virus in the maize was not evident. Chauhan (1985) showed that slow germinating seeds had a high incidence of seedborne MDMV. So as not to confuse results, only fast germinating seeds were selected for planting, thus reducing the chance of the occurrence of seed-transmitted virus. Knox (1983) showed that CMV was seedborne in some seed sources. Seed-transmitted CMV was not evident in the maize plants tested in the current exercise, possibly because not a large enough number of plants were tested and an effort was made to select virus-free seed sources for experimental purposes.

The sequence of sap-inoculations of viruses appeared to be important (Table V.2). When MDMV was inoculated first, followed after a time interval by CMV, both viruses could

replicate together in the same plant, even if MDMV had been multiplying for 6 days in the host before CMV was inoculated. However if CMV was sap-inoculated first, subsequent infection by MDMV was less efficient. Fewer plants were doubly infected with both viruses if CMV was allowed to establish itself in the host before MDMV was sap-inoculated. Thus CMV in some way interferred with the MDMV infection process. This type of interference has been found in other situations involving other viruses (see Chapter II, Literature Review). This mechanism of interference could possibly operate in nature and may be a reason why so few clear cut MDMV infections are visibly encountered. For clarification, this observation will have to be further investigated. Ideally the strain of CMV which occurred in the natural, mixed infected maize should have been used for studying the interaction of CMV and MDMV in the laboratory experiments. Attempts to separate and purify the two viruses from the natural double infection were not made. Rather than spend time on this, CMV-K was selected for the investigation as it had been previously reported to infect maize (Rao and Francki, 1982). In addition, results indicated that it gave distinct symptoms in maize-A (Figure IV.1.c) and high yields of virus could be extracted when this host was infected.

While compiling the results for this work, it became evident that MDMV-B-ST propagated and extracted from maize cv.KEP, also contained spherical particles (Figure IV.8).

IEM using anti-MDMV-B-ST serum also revealed that this antiserum could recognise spherical particles and when used in IEB, it would probe the CMV protein. The possibility that the MDMV-B-ST isolate used in this work contained CMV must therefore be considered. The decorating technique applied to a leaf-dip preparation from a doubly infected leaf (Figure V.1) raised further questions about CMV contamination of the MDMV-B-ST isolate. Virus particles from plants infected with a mixture of viruses were trapped using anti-MDMB-B-ST and anti-CMV-K serum, mixed 1:1, and decorated using anti-MDMV-B-ST serum. Spherical particles were observed to be closely associated with the filamentous particles. It was too late in the programme at that point to separate the MDMV from the spherical virus component which constituted, in all probability, seedborne CMV. In retrospect the presence of CMV could explain some of the results of the sap-inoculation experiments. It has been mentioned that if CMV is present first and is subsequently challenged by MDMV-B-ST, the multiplication of the other appears to be impeded. However, if the MDMV-B-ST also contained a low concentration of CMV and it was a different strain to CMV-K, then a cross-protection mechanism between the two CMV strains could come into action (see Chapter II, Literature Review). It would be interesting to see if this work was repeated by using an MDMV-isolate free of CMV, if the same results could be obtained.

2. Aphid transmission of mixed infections

It was important to investigate the transmission of MDMV and CMV when they occurred singly and also together as a mixed infection in maize. This would perhaps help to understand some of the epidemiological aspects of mixed infections in the field situation.

Aphid tranmission experiments revealed that CMV-K could be transmitted efficiently from infected squash and maize to uninfected plants by three aphid species namely *R. maidis*, *R. padi* and *M. persicae* (Table V.4). *M. persicae* appeared to be the most efficient vector of the three aphid species for transmitting CMV from one maize plant to another. The *R. maidis* aphid does not normally colonise squash; maize is its preferred host. From these experiments it was evident that the aphid needed only to probe the infected plant tissue for acquisition of the virus and subsequent transmission. Similarly MDMV-B-ST could be transmitted by all three aphid species tested.

It was particulaly important to test the plants on which the aphids had been maintained to ensure that, if collected in the field, the aphids had not fed previously on CMV-infected plants. In one case, *M. persicae* aphids collected in a city garden were shown to transmit CMV to the plants in the laboratory on which they were propagated. The aphid colony had to be destroyed.

Both CMV and MDMV could be transmitted from doubly infected plants to uninfected maize plants. M. persicae seemed to be more efficient at transmitting the CMV component of the mixed infection whereas R. padi transmitted MDMV more efficiently. M. persicae is known to transmit CMV particularly well compared to other aphid species (Francki et al., 1979). This aphid occurs commonly in vegetables, ornamentals and other agricultural crops. If these plants were grown in the vicinity of maize crops, or alternated with them, the presence of M. persicae could contribute largely to the overall epidemiology of CMV in maize.

The colonizing behaviour of the aphids may affect the efficiency of their transmission of the two viruses. R. maidis feeds mainly on the young unfolding leaves and tassle of the infected plant whereas R. padi prefers to feed on the stem region of the maize plant. This could explain the difference in the efficiences with which the two aphids transmitted MDMV-B-ST. This aspect was not investigated further. The colonizing behaviour of M. persicae on maize was not observed.

D. MAIZE PROTOPLASTS AND THEIR INFECTION WITH CMV AND MDMV

As mixed infections of maize with MDMV and CMV could occur in the natural field environment and could also be maintained in laboratory conditions, it was of interest to study mixed infections of maize protoplasts with these two viruses. In similar studies involving other viruses, phenomena such as interference and cross-protection were demonstrated (see Chapter II, Literature Review).

Leaf mesophyll protoplasts isolated from maize plants were used since these simulated the condition in the whole plant more accurately than maize cell suspension cultures. However there are advantages to using suspension cells. They can easily be maintained in culture under controlled conditions for long periods so that an ongoing supply of cells is readily available for experimental work. Since mesophyll protoplasts can only survive in suspension for approximately 70 hours, suspension cultures are being preferentially used for genetic manipulation experiments in maize (H. Lorz, pers. comm.). For the present study of virus infection of maize protoplasts, mesophyll cells were used .

It was evident that the ease with which protoplasts were produced varied for different maize cultivars. After screening several maize types, maize-A was selected for use in this investigation, as it was a good host for both MDMV and CMV. It expressed clear symptoms when infected with either of these viruses and it had an epidermis which could be easily removed,

thus facilitating the preparation of protoplasts. Some of the maize types tested were particularly recalcitrant to protoplast production (Table VI.2). Even though maize-A appeared to be the most suitable, the quality of different batches of protoplasts varied considerably. It has previously been found with protoplasts of other plant species that the number of intact, viable protoplasts produced depends to a large extent on the physiological state of the plant at the time of using the leaves for protoplast isolation (Okuno and Furusawa, 1976).

Initially infection of protoplasts with one virus i.e. either CMV or MDMV, was done. It was noticeable that appreciably higher concentrations of CMV-K in the inoculum were required for infection of maize protoplasts than had previously been reported for other protoplast systems (Table II.4). This could be explained when one considers the instability of the CMV-K particles (Figure IV.2.c). To obtain particles in an intact, infectious state i.e. with all RNA's present, large amounts would be necessary in the inoculum. It was essential for successful infection of protoplasts that freshly purified CMV was used. This necessitated protoplast isolation, virus purification and protoplast infection on the same day. All complementary types of RNA of this multicomponent virus would have to infect the protoplast simultaneously. The infection curve for both CMV and BMV infection of maize protoplasts was similar to that obtained by Okuno et al. (1977) for infection of barley protoplasts with BMV. However it appeared that less time was required for the CMV particles to enter the maize protoplasts and start multiplying when compared to that required by BMV in barley protoplasts.

Although several attempts were made, infection of maize protoplasts with CMV-Y and CMV-S, was unsuccessful. It would have been interesting to monitor the survival of these protoplasts to ascertain the reasons why infection did not take place. Hypersensitivity to infection of protoplasts by viruses has been noted in some cases (Otsuki et al, 1972). In addition, these workers showed that when tobacco protoplasts were infected by a necrosis forming strain of TMV, the N genes for the necrotic reaction were not switched on in the protoplasts.

Fluorescent-labelled antibodies to detect the presence of CMV-K in infected protoplasts required further work to minimize background fluorescence (Figure VI.6) and to quantitate results. A time course study using FITC-labelled antibodies by the direct and indirect staining procedures could have been used to compare these results with those obtained by DAS-ELISA. By using two fluorescent stains with different emission wavelengths it would have been possible to quantitate the number of protoplasts infected with either MDMV or CMV , or both simultaneously.

Radiolabelling protoplasts with ^{35}S-methionine proved to be more difficult than it had at first appeared. Labelled host proteins obscured the detection by autoradiography of any viral-induced proteins (Figure V.7). Treatment of protoplasts with UV irradiation or inclusion of actinomycin in the incubation media have previously been shown to reduce host protein production. The conditions for these treatments vary

considerably for different protoplast systems. There is a fine balance between the reduction of the host proteins and the total destruction of the protoplasts. This aspect needed further investigation in the maize protoplast system to obtain conclusive results.

Several attempts to infect the maize protoplasts with MDMV-B-ST were made before this was successful. This was mainly due to the sensitivity of the protoplasts to $CaCl_2$ treatment. Once a concentration was found which allowed the permeability of the membrane to alter sufficiently for MDMV-B-ST to enter the cells, without destroying them, an infection curve could be investigated. MDMV-B-ST appeared to multiply at lower levels and more slowly than CMV in the protoplasts.

Once infection of the maize protoplasts had been achieved using either CMV or MDMV singly, the next step was to inoculate the two simultaneously. In such double infections only CMV was found to infect the protoplasts. This could imply that interference in the infection process and replication of MDMV occurred, as was apparent in whole plants. Alternatively the greater concentration of CMV in the inoculum (50 ug/ml) compared to MDMV (2 ug/ml) could mean that CMV occupied all infection sites thus excluding MDMV to such an extent that it was not detectable. It would have been interesting to allow the one virus to establish itself within the protoplasts before inoculating with the second virus to see whether a similar

interference mechanism existed. This aspect was investigated to some extent by isolating protoplasts from systemically infected plants and inoculating them with a second virus. IEB was used initially to assay the protoplasts from the systemically infected plants for the presence of viruses since this technique is more sensitive than ELISA (Rybicki and von Wechmar, 1983) and it was not known what the concentration of virus in the protoplasts would be. Both methods could detect virus particles in the protoplasts. It must however be noted that probably not all protoplasts isolated from infected plants contain virus particles. This could have been assessed by fluorescent-labelling experiments. In addition after inoculation, if protoplasts from systemically infected leaves were inoculated with the second virus, it would have been interesting to ascertain how many of the cells contained both viruses simultaneously and how many had only one or the other. This could have been achieved using FITC-labelled antibodies.

When protoplasts were isolated from CMV-infected seedlings no infection by MDMV could occur. Again this result correlates to the findings with whole plants. In the latter incidence, when plants were sap-inoculated with CMV, followed after a time interval by MDMV, less plants were shown to be infected by both viruses simultaneously. It must however be noted that if the MDMV-B-ST isolate contained a low concentration of CMV as suspected, this could have influenced the results of these experiments. If time had permitted, it would have been interesting to repeat the protoplast infection using a pure MDMV isolate.

The survival of protoplasts isolated from seedlings infected with either CMV, MDMV or both (VI.H) showed that a hypersensitive reaction to infection by these viruses was not expressed. The protoplasts isolated from leaves containing both MDMV and CMV appeared not to be seriously affected by the presence of the viruses. Again it must be remembered that probably only a small proportion of the protoplasts contained both MDMV and CMV simultaneously in one cell. The actual number of protoplasts containing either CMV or MDMV could have been quantitated using fluorescent-labelled antibodies prepared against the two viruses. This was not done as it was envisaged that standardizing conditions would have taken considerable time.

The results of experiments involving infection of protoplasts were difficult to analyse and validate statistically. In most cases, the graphs were plotted using mean values of three independent experiments. Standard deviations cannot be calculated accurately using only three values (Leaver and Thomas, 1979). It was decided that for purposes of this work only trends need be represented graphically and the results obtained by DAS-ELISA for the presence of virus and survival counts indicated these trends.

E. LIPOSOMES

Liposome-mediated transfer of virus particles and their RNA has been shown to often enhance the efficiency of infection (Fukanaga et al., 1981; Fraley et al., 1982, 1983; see Chapter II). With this in mind the possibility of infecting protoplasts with CMV and/or MDMV via liposome fusion was considered. Different types of liposomes, varying in their encapsulating volume and charge were produced and fluorescent dyes were incorporated into them. These could be fused to the protoplasts via a PEG-mediated process. Attempts to encapsulate CMV-K into the liposomes were not successful. It appeared that the particles associated with the liposomes' surface but did not become encapsulated. There are several explanations for this. To establish a protocol for encapsulation, it probably would have been better to initially use a more stable virus than CMV. The charge of the virus is an important aspect. If incorrect, repulsion between the virus particles and the liposomes will occur. This can be mediated to some extent by the type of buffer used for suspension of the virus particles. The charge carried by the liposomes themselves can also be altered depending on the type of phospholipid used. Virus particles which had not associated with the liposomes were removed by gradient centrifugation. The liposomes were then assayed for the presence

of virus by disrupting them using a detergent so that their contents were released, which were then tested by DAS-ELISA. Although efficiencies of encapsulation of viruses may be only 2-10%, the results obtained by DAS-ELISA indicated that the virus particles appeared to be mainly adsorbing to the liposome surface rather than becoming encapsulated. ^{35}SLR-labelled-CMV did not appear to be associated with the liposomes when analysed by autoradiography. It could be that when labelled, the charge carried by the virus is altered so that no adsorption to, or incorporation into, the liposomes is possible.

Since incorporation of CMV-K into liposomes was not successful, fusion of the liposomes to protoplasts appeared futile at the time and this aspect of the programme was discontiued.

Concurrent with the production of liposomes, isolation of RNA from both CMV and MDMV was initiated (Figures IV.3(a) and 8(a)). Encapsulation of the RNA of these viruses and subsequent infection of protoplasts via liposomes was envisaged. The proteins produced in protoplasts when they had been inoculated with either MDMV- or CMV-RNA could have been analysed and the interference mechanism which appeared to be operational between the two, further investigated. The extreme sensitivity of RNA to

RNases would have necessitated sterile techniques. In addition, the charge of the liposomes, used for encapsulation of the RNA, would have to be correct and would have to allow for fusion of the liposomes to the protoplasts.

F. CONCLUSION

Evidence is presented that MDMV and CMV occur in natural mixed infections in field grown maize in three geographically distant regions. Electron microscopy, virus transmission and serological tests were used to identify the viruses.

To simulate natural field infections and to study such mixed infections in the laboratory, isolates of MDMV and CMV were used to sap-inoculate maize plants. The inoculum consisted of either a mixture of viruses or single viruses inoculated sequentially i.e. one virus followed after an interval by another. It was shown that the two viruses could co-exist in individual plants when they had been inoculated simultaneously. However the presence of CMV in the maize plants appeared to interfere with, or inhibit, subsequent multiplication by MDMV. A cross-protection mechanism could be in operation in this case. The phenomenon of cross-protection is not fully understood and several explanations have been proposed for it.

Aphid transmission experiments confirmed that both MDMV and CMV could be transmitted from singly and doubly infected maize. In the case of CMV, aphids could transmit it from squash, a host which is not normally colonized by the maize aphid, R. maidis. Transmission from maize which was infected with both MDMV and CMV, to uninfected maize was also successful. This findings could be important epidemiologically in the natural field situation particularly if seedborne virus is present.

Maize protoplasts were isolated and successfully infected with CMV and MDMV. The infection curves for each virus were compared. When the two viruses were inoculated together, a similar situation to that in the whole plant resulted i.e. MDMV infection of the protoplasts was inhibited by the presence of CMV. Although DAS-ELISA appeared to be a sensitive and accurate method for detection of the viruses in the protoplasts, other assay techniques such as detection by fluorescent-labelled antibodies and ^{35}S-methionine-labelling of proteins were attempted. Protoplasts were prepared from maize seedlings which had previously been sap-inoculated with MDMV and/or CMV. These were super-infected with either MDMV or CMV. Protoplasts, some of which already contained CMV, could not be subsequently infected with MDMV. These results correlate with those obtained for sap-inoculation of whole plants.

Liposomes were produced. Fluorescent dyes were encapsulated in them and they were subsequently fused to maize protoplasts. Efforts to incorporate CMV-K into different types of liposomes were not successful, possibly because of the instability of the virus concerned or the method of preparation of the liposomes which was used.

CHAPTER IX

MATERIALS AND METHODS

A. BUFFERS AND REAGENTS

Chemicals used for buffers were either Merck or Laboratory and Scientific products.

1) Standard buffers

Standard buffers were made according to Williams and Chase (1968). In all cases 0,1 M buffers were prepared as stock solutions and these were diluted when necessary.

a) Potassium phosphate buffers

Solution A: $KH_2 PO_4$; 0,5 M (M_r = 136,09). Dissolve 68,04 g in a final volume of 1 litre of distilled water.

Solution B: $K_2 HPO_4$, 0,5 M (M_r= 174,18). Dissolve 87,09 g in a final volume of 1 litre of distilled water.

These two stock solutions were mixed as follows according to the pH of the buffer required.

pH	Solution A (ml/litre)	Solution B (ml/litre)
5,8	184,0	16,0
6,0	175,4	24,6
6,5	137,0	63,0
7,0	78,0	122,0

b) Phosphate buffered saline

Mix 1 part 0.15 M NaCl (M_r = 58,44) with 1 part 0,1 M potassium phosphate buffer (pH 7,0) (IX.A.(a)).

(2) Virus purification and storage buffers

a) 0,1 M sodium phosphate buffer

This buffer was required for extraction of cucumber mosaic virus by the method of Mossop et al. (1976).

Solution A: $Na\ H_2PO_4$; 0,2 M
Dissolve 27,6 g of $NaH_2PO_4.H_2O$ in a final volume of 1 litre of distilled water.

Solution B: $Na_2\ HPO_4$; 0,2 M
Dissolve 28,4 g of $Na_2\ HPO_4$ in a final volume of 1 litre of distilled water.

These two stock solutions were mixed as follows according to the pH of the buffer required.

pH	Solution A (ml/litre)	Solution B (ml/litre)
6,0	438,5	61,5
8,0	26,5	473,5

b) Borate buffer

Solution A: boric acid; 0,2 M

Dissolve 12,4 g in a final volume of 1 litre of distilled water

Solution B: sodium tetraborate.$10H_2O$; 0,05 M

Dissolve 19,05 g in a final volume of 1 litre of distilled water.

These two stock solutions were mixed as follows according to the pH of the buffer required.

pH	Solution A (ml/litre)	Solution B (ml/litre)
8,0	700	300
9,0	200	800

c) 0,1 M acetate buffer

The following stock solutions were made:

Solution A: Sodium acetate; 2,0 M

Dissolve 227,16 g $CH_3COONa_3H_2O$ in a final volume of 1 litre of distilled water.

Solution B: Acetic acid; 3,5 M

200 ml glacial acetic acid/litre

Solution C: NaCl; 5,0 M

Dissolve 292,2 g in a final volume of 1 litre of distilled water.

For pH 6,0 mix 20 ml solution A, 3,7 ml solution B and 32 ml solution C and make up to two litres with distilled water.

d) 0,1 M sodium phosphate/thioglycollic acid/DIECA (pH 8,0).

This buffer was used for the extraction of virus from infected plant tissue and for crushing infected leaves for sap-inoculation of plants.

Mix solutions A and B of 0,1 M sodium phosphate thioglycollic acid and 0,1% (w/v) DIECA. Make up to 900 ml, adjust pH using 0,1 M NaOH and then make up to 1 litre with distilled water.

3) Enzyme-linked immunosorbent assay (ELISA)

Buffers and Reagents

a) Antibody dilution buffer

Phosphate buffered saline (IX.A.4.(b)) was used for all antibody dilutions.

b) Rinsing buffer (PBS-Tween)

Used for washing plates between coating. Phosphate buffered saline (IX.A.1.(b)) containing 0,05% (w/v) Tween-20 (Merck) and 0,05% (w/v) sodium azide.

c) Post-coating and conjugate dilution buffer (PBS-Tween-BSA).

Phosphate buffered saline (IX.A.1(b)) containing 0,2% (w/v) bovine serum albumin (Bayer-Miles Pty. Ltd), 0,05% (v/v) Tween 20 and 0,05% (w/v) sodium azide.

d) Substrate buffer (10% diethanolamine).

This buffer was made according to the specifications of Clark and Adams (1977). For 1 litre of substrate buffer the following was mixed.

97 ml	diethanolamine (Merck)
800 ml	H_2O
0,2g	sodium azide

The pH was adjusted to pH 9,8 with concentrated HCl and made up to a final volume of 1 litre with distilled water.

e) 4-nitrophenylphosphate (Merck) was used at a concentration of 1,0 mg/ml in substrate buffer (IX.A.3.(d)).

4) Polyacrylamide gel electrophoresis buffers

These buffers were made according to Laemmli (1970).

a) Resolving gel buffer

1 M Tris-HCl pH 8,8

Dissolve 60,6 g Tris-HCl (Merck) in 400 ml distilled water. Adjust to pH 8,8 with approximately 7 ml HCl. Dilute to a final volume of 500 ml.

b) Stacking gel buffer

1 M Tris-HCl pH 6,8

Dissolve 60,6 g Tris-HCl (Merck) in 400 ml distilled water. Adjust to pH 6,8 with HCl. Dilute to a final volume of 500 ml.

c) Running buffer

Dissolve 30,3 g Tris-HCl
141 g Glycine
10 g sodium dodecyl sulphate (SDS)

This will make buffer at ten times strength. Dilute with distilled water when needed.

d) Acrylamide stock solution

30% acrylamide : 0,8% bis-acrylamide.

Dissolve 150 g acrylamide (Merck) and 4,0 g bis-acrylamide (BDH Chemicals) in a final volume of 500 ml water. Store at 4°C.

e) Dissociation mixture

10% SDS, 10% B-mercaptoethanol, 15% glycerol, 0,01% bromophenol blue in 1 M Tris pH 6,8

Dissolve	10 g	SDS
	10 ml	B-mercaptoethanol (BDH Chemicals)
	15 ml	glycerol
	5 ml	0,2% (w/v) bromophenol blue

Make up to a final volume of 100 ml with distilled water.

f) 10% sodium dodecyl sulphate (SDS)

Dissolve 10 g of SDS in a final volume of 100 ml of distilled water.

g) 1,5% ammonium persulphate

Dissolve 1,5 g ammonium persulphate (Merck) in 100 ml H_2O. Should be freshly prepared.

h) Temed

N,N,N^1,N^1 Tetramethylethylendiamine ($C_6H_{16}N_2$) (Merck)

i) Coomassie stain

This was prepared as follows-

0,2% (w/v) Coomassie brilliant blue (Merck)

45% (v/v) methanol

10% (v/v) glacial acetic acid

j) Destain

This was prepared as follows-

25% (v/v) methanol

10% (v/v) acetic acid

65% (v/v) distilled water

5) Immuno-electroblotting buffers

These were prepared according to the specifications of Rybicki and von Wechmar (1982).

a) Transfer buffer

pH 8,3

25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol.

Dissolve: 30,29 g Tris

144 g glycine

in a final volume of 1 litre of distilled water. Dilute 10 fold when needed. Required 5 litres for apparatus i.e. 500 ml buffer, 1 litre methanol made up to a final volume of 5 litres.

b) Soaking buffer

1% bovine serum albumin (Bayer-Miles Pty. Ltd) dissolved in 10 mM Tris HCl/saline pH 7,4. This is used for soaking electroblots. Also used for diluting antisera and goat anti-rabbit horseradish peroxidase.

c) Washing solutions

0,15 M saline containing 0,05% (v/v) Tween 20.

d) Enzyme-substrate solution

Substrate buffer

0,05 M Tris-HCl; 0,2 M NaCl

Dissolve: 6,06 g Tris-HCl

11,69 g NaCl

in final volume of 1 litre of distilled water.

Substrate

3 mg/ml horseradish peroxidase colour development reagent (4 chloro-1-napthol) (Biorad Laboratories, EIA purity grade) was dissolved in chemically pure methanol (BDH chemicals). This is light sensitive and was stored in the dark. When required 1 volume of substrate was added to 5 volumes of substrate buffer (see above). To this 0,015% (v/v) hydrogen peroxide (Merck, stored at $4^{o}C$) was added.

6) Buffers used for isolation of maize protoplasts

a) Enzyme digestion medium

2% cellulase, 0,5% bovine serum albumin, 0,7 M mannitol.

Dissolve: 0,2 g cellulase "Onozuka" R-10 (Yakult Pharmaceutical Industry Co. Ltd.)

0,05 g BSA

1,27 g D-mannitol (Merck, M_r = 182,17)

in a final volume of 10 ml of distilled water.

b) Washing solution

0,7 M mannitol

Dissolve 127 g D-mannitol in a final volume of 1 litre of water. Filter sterilize by passing through Sartorius membrane filters of pore size 0,2 nm (type SM66) or Millipore filter unit Millex GS or HA (Millipore South Africa Ltd).

c) Incubation Medium

This was prepared according to Okuno et al., 1977).

0,2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 10 mM $CaCl_2$, 1 uM KI, 0,01 uM $CuSO_4$

0,7 M mannitol

Filter sterilize before use.

500 ug/ml cephaloridine (Sigma)

d) 0,02 M sodium citrate (pH 5,6)

Dissolve 0,588 g of sodium citrate (Merck, M_r= 294,10) in 80 ml of distilled water. Adjust pH and make up to 100 ml with distilled water.

7) Fluorescent antibody conjugating and staining buffers

a) Conjugation buffer

0,02 M sodium carbonate, pH 9,8

This buffer was used for the conjugation of fluorescein iso-thiocyanate (FITC) to the gamma globulins. The stock buffer was prepared according to the specifications in buffer No. 19 of Williams and Chase (1968).

Stock solutions

Solution A: Sodium acid carbonate, 1M. Dissolve 84,0 g of $NaHCO_3$ in a final volume of 1 litre distilled water.

Solution B: Sodium carbonate, 1M. Dissolve 106,0 g Na_2CO_3 in a final volume of 1 litre of distilled water.

Stock buffer: 1M sodium carbonate, pH 9,8. Mix 34,3 ml of Solution A with 21,9 ml of Solution B. Dilute to 1 litre with distilled water. To make 0,02 M sodium carbonate buffer pH 9,8, the stock solution was diluted 1:50 with distilled water.

b) Rinsing buffer

Phosphate buffered saline pH 7,0 (IX.A.1(b))

c) Coating solution

0,1% (w/v) ovalbumin (Miles Laboratories Pty. Ltd.)in distilled water.

d) Mounting fluid

80% (v/v) glycerol in 0,02 M sodium carbonate buffer pH 9,8.

8) Buffers used for the preparation of liposomes

a) Liposome buffer

5 mM Tris; 50 mM NaCl; 1 mM EDTA, 0,7 mM mannitol (pH 7,6).

Dissolve: 0,06 g Tris
0,29 g NaCl
0,04 g EDTA
12,7 g mannitol in a final volume of 100 ml of distilled water.

b) HEPES buffer

1 M HEPES (Sigma M_r=260,3)

Dissolve 26,03 g in a final volume of 100 ml of distilled water.

c) Fusion medium

5 mM Tris; 50 mM NaCl; 0,7 M mannitol

Dissolve: 0,06 g Tris
0,29 g NaCl
12,7 g mannitol in a final volume of 100 ml of distilled water.

9. Buffers used for extraction of RNA.

a) Disruption buffer:

0,2 M Tris HCl (pH 8,25); 0,2 mM EDTA; 2% (w/v) SDS.

Dissolve 12,1 g Tris HCl (M_r= 121,14), 0,037 g EDTA (M_r= 372,24) and 10 g SDS (M_r= 288.4) in a final volume of 500 ml of sterile distilled water.

b) Running buffer: (TBE)

0,089 M Tris HCl; 0,089 M boric acid; 0,5 M EDTA (pH 8)

Dissolve 27 g Tris HCl, 13,75 g Boric acid and 10 ml of 0.5M EDTA (pH 8,0) in a final volume of 1 litre of sterile distilled water. When required, dilute one volume with four volumes of sterile distilled water.

c) Resuspension mix:

100 ml TBE, 5% (v/v) glycerol, 0,1 g bromophenol blue.

d) 3 M Sodium Acetate (pH 5,5)

Dissolve 24,6 g sodium acetate (M_r = 82,03) in a final volume of 100 ml of sterile distilled water.

e) Phenol

Phenol was stored at -20°C and when required melted at 65°C. 8-hydroxquinoline was added to a final concentration of 0,1%. This yellow compound imparts a yellow colour to the phenol thus enabling the phenolic phase to be easily recognised. The melted phenol was extracted with an equal volume of buffer (1,0 M Tris, pH 8,0) and then with 0,1 M Tris (pH 8,0) and 0,2% B-mercaptoethanol. The pH of the aqueous phase was adjusted to pH 7,6 and the preparation stored at 4°C until use in RNA extraction.

f) 1,5% Agarose

Dissolve 1,5 g agarose (Sigma, Type I) in 100 ml of TBE.

g) Destain

0,01 M $MgCl_2$.

10. Buffers for Electron microscopy

a) Negative stain

2% (w/v) Uranyl acetate pH 5,0.

B. ANTISERA

The following antisera were used in the dissertation and were made available from the Departmental collection.

Antisera	Optimal concentrations IgG/conjugate
anti-BMV-ST	1/500
anti-CMV-Is	1/400
anti-CMV-Q	1/250
anti-CMV-S	1/400
anti-CMV-Tob	1/400
anti-CMV-Y	1/400
anti-MDMV-B	1/300
anti-MDMV-B-ST	1/300

C. PLANT MATERIAL

The following hosts were used in the dissertation. Only common names will be used in the text.

Host	Abbreviation/code
Chenopodium quinoa	*C. quinoa*
Cucurbita pepo L. var. Long White bush	*C. pepo*
Nicotiana clevelandii	*N. clevelandii*
Nicotiana glutinosa	glutinosa tobacco
Nicotiana tabacum cv. Soulouk	Soulouk tobacco
Nicotiana tabacum xanthi	*N. tabacum*
Zea mays cv. Kalahari Early Pearl	cv. KEP
Zea mays cv. PNR 95	Maize-A
Zea mays cv. SNK hybrid	Maize-B
Zea mays cv. SNK hybrid	Maize-C
Zea mays cv. SA 100	Maize-D
Zinnia elegans cv. Dahlia coloured mix	Zinnia

D. METHODS

All experimental work was carried out in laboratory conditions. The temperature was controlled and was 22°C at all times.

1. Maintenance, propagation and source of virus isolates

a) Virus Sources

(i) Cucumber mosaic virus

Cucumber mosaic virus strain-K was made available from the Departmental virus collection. The virus was propagated from dried leaf tissue which had been stored at 4°C over dry self-indicating silica gel. This virus strain was originally obtained from Dr R.I.B.Franki of the Waite Institute, Adelaide, Australia.

(ii) Maize dwarf mosaic virus

The maize dwarf mosaic virus isolate used in this study was propagated in maize cv. Kalahari Early Pearl (IX.C) as reported by R. Chauhan (MSc thesis, 1985).

b) Storage of viruses

(i) Short term storage

Infected plant material was stored at 4°C in sealed plastic bags before extraction of the virus.

(ii) Long term storage

Infected leaves were desiccated over self-indicating silica gel under vacuum. The dried material was subsequently stored in vials between layers of dry self-indicating silica gel at 4°C. Desiccated material was used as a stock of original virus isolate and stored to ensure that the same virus was available for later use.

For long term storage, fresh infected leaf material was placed in small cryostat vials and placed in a cryostat (-170°C)

c) Maintenance and propagation of viruses

Cucumber mosaic virus strain-K (CMV-K) was propagated in either glutinosa tobacco or squash. The seed of N. glutinosa was originally obtained from Dr. Bar Joseph, Volcani Institute, Israel. Dried infected tissue was used to start the first infection. The leaves were ground up in 0,05 M sodium phosphate pH 7,0 containing 0,1% thioglycollic acid and 0,1% DIECA (IX.A.2 (d)).

The ground pulp was filtered through cheesecloth, a pinch of celite added and inoculated immediately onto tobacco and/or squash. Fresh infected glutinosa tobacco was used as inoculum for further propagation. Squash was inoculated at the early dicotyledonous stage and tobacco at the four leaf stage.

Glutinosa tobacco had certain advantages over squash for an ongoing supply of fresh infected plant material.

(i) Infected glutinosa tobacco could easily be maintained for up to 3 months in a position with low light supply (i.e. under plant room tables), without decline.

(ii) In a programme with CMV, particular care is essential to control aphid infestation. Glutinosa is unattractive to aphids used in other projects.

(iii) Glutinosa tobacco is a local lesion host for tobacco mosaic virus (TMV) and would be revealed if it were present. A mixed infection of CMV and TMV on Nicotiana tabacum would not be distinguishable by symptoms only.

(iv) Different strains of CMV are distinguishable by the symptoms which they produce on glutinosa tobacco (see Table IV.1).

Maize dwarf mosaic virus (strain-B) seed-transmitted (MDMV-B-ST) was propagated from infected maize leaves to maize seedlings at the

three leaf stage. These maize seedlings were grown only from fast-germinating seed to ensure uniform, strong seedlings (Chauhan, 1985). In addition it had been shown earlier that fast-germinating seed contained less seedborne MDMV and CMV and therefore had the added advantage of selecting predominantly virus-free seedlings (von Wechmar and Chauhan, 1984).

d) Plant growth room conditions

Inoculated plants were maintained in a plant growth room under the following controlled conditions:

(i) 14 hour light/10 hour dark cycle.

(ii) approximately 70% humidity.

(iii) average daylight temperature of 24°C day/ 21°C night temperature cycle.

2. Centrifugation

Low speed centrifugation (L.S.), unless otherwise stated was carried out at 8000rpm for 10 minutes in a Sorvall RC 2-B or RC-5 refrigerated centrifuge using Sorvall SS-34, GSA or GS-3 rotors. The relative centrifugal forces, in gravities, exerted by the SS 34, GSA and GS-3 rotors at 8000 rpm were 7265g, 10444g and 10825g respectively.

High speed centrifugation (H.S.) was at 34000rpm for 90 minutes unless otherwise stated in Beckman type 35, 50Ti and 60Ti rotors. Beckman L3 -50 and L5 - 65 ultracentrifuges were used. The relative centrifugal forces for the 35, 50Ti and 60Ti rotors were 142800g, 226400g and 361300g respectively at 34000rpm.

3. Virus purification

a) Cucumber Mosaic Virus Strain-K (CMV-K)

The procedure used was that of Mossop et al (1976) and P. Lupuwana (1985) and is summarized below:

To obtain good virus yields, infected plants were harvested 7 - 10 days after inoculation. The fresh leaves were homogenized in a Waring blender with 0,1 M sodium phosphate buffer (pH8) (IX.A.2(b)) containing 0,1% (v/v) DIECA (diethyldithiocarbonate) and 0,1%(v/v) thioglycollic acid. The leaf weight to buffer volume ratio was approximately 1:2. After filtering the pulp through cheesecloth, the supernatant was subjected to L.S. centrifugation. The resulting supernatant was mixed with Triton

X-100 (BDH Chemicals, England) to a final concentration of 2% and stirred at 4°C for 15 minutes. The H.S. pellet was resuspended in 0,1 M sodium phosphate (pH 8) (IX.A.1(a)) to 1/30 of the original volume. Another cycle of L.S. and H.S. centrifugation was carried out. The final pellet was resuspended in:-

(i) 0,005M borate buffer (pH 9,0) (IX.A.2(b)) and dialysed against two litres of the same buffer containing 2% formaldehyde (FA) to stabilize the virus and prevent dissociation. This product was used for immunization of rabbits (see IX.D.4).

(ii) 0,02M potassium phosphate buffer (pH 5,6) (IX.A.1(a)) for infecting protoplasts.

b) Maize dwarf mosaic virus

MDMV-B-ST was purified by the method of Lamy et al. (1979) adapted by Chauhan (1985). Briefly the method was as follows:

Infected leaves were homogenized in 0,1M potassium phosphate buffer (pH 7,0) (IX.A.1(a)) containing 0,01M EDTA and 1% (w/v) sodium sulphite in a ratio of 1:1 (w/v) buffer to leaves. The pulp

was filtered through cheesecloth and subjected to L.S. centrifugation. The supernatant was mixed with 10% (w/v) polyethylene glycol (PEG) (Hoechst Chemicals, M_r=6000) and 3% (w/v) sodium chloride. The resulting precipitate was collected by L.S. centrifugation and resuspended in 0,1M potassium phosphate buffer (pH 7,0) (IX.A.1(a)) to 1/10 the original volume. Non-resuspended precipitate was removed by L.S. centrifugation and the virus pelleted from the remaining supernatant by H.S. centrifugation. The resulting pellet was resuspended in 0,1M Potassium phoshate buffer (pH 7,0) (IX.A.1.(a)).

c) Brome mosaic virus

BMV-ST was used in preliminary investigation of the infection of maize protoplasts. The procedure used is summarized below:

The leaves of BMV-infected barley plants (Old Clipper) were homogenized in acetate buffer, pH 6,0 (IX.A.2.(c)). The pulp was filterd though cheesecloth and subjected to L.S. centrifugation. The supernatant was mixed with 7,5% (w/v) PEG and 2,5% (w/v) sodium chloride and non-resuspended

precipitate removed by L.S. centrifugation. The resulting precipitate was resuspended in acetate buffer (pH 6,0) subjected to H.S. centrifugation and the pellet resuspended in 0,02 M sodium citrate buffer (pH 6,0) (IX.A.6.(d)). The resuspended pellet was subjected to L.S. centrifugation and the supernatant used for protoplast infection.

d) Quantitation

Virus preparations were quantified by spectrophotometry. Virus suspensions were diluted in the appropriate buffer and absorption at 260nm and 280nm recorded on a Beckman Model 25 spectrophotometer. The extinction coefficient $E^{0,1\%}_{260nm}$ = 5,0 was used for CMV (Francki et al, 1979). $E^{0,1\%}_{260nm}$ = 2,7 was used for MDMV-B-ST (Langenberg, 1973).

4. Production of antisera

a) Preparation of antisera

Virus preparations that had undergone at least two H.S. centrifugation cycles were mixed with an equal volume of Freund's incomplete adjuvant. Rabbits were immunized once weekly for three weeks - with a booster injection at 6 weeks, followed by monthly boosters.

After 6 weeks the rabbits were bled weekly from a marginal ear vein. Twenty to thirty millilitres of blood were collected and allowed to clot at 4°C overnight. The serum fraction was then separated by L.S. centrifugation.

Bleedings were collected and titred using the indirect ELISA method (IX.D.5.(a)(ii)). Those bleedings which showed the highest titre were pooled and host absorbed for IgG preparation (IX.D.4(c)).

Rabbits immunized with CMV were injected with CMV-FA. This was prepared as given in (IX.D.3(a)). Before injecting the final concentration of FA was adjusted to 0,2% by dialysis.

b) Host Absorption of antisera

The method of Erasmus (1982) and Chauhan (1985) was used and summarized below:

Leaves from healthy host plants were ground in 1:1 (w/v) ratio in 0,05 M potassium phosphate buffer (IX.A.1(a)). The serum to be host absorbed was mixed with this sap in a ratio of 2 volumes serum to 1 volume sap. This mixture was left at room temperature overnight. The precipitate was removed by centrifugation at 15000 rpm for 15 minutes and the supernatant retained. The above absorption procedure was repeated with a smaller volume of sap.

To the supernatant, saturated 4M ammonium sulphate (Merck) was added dropwise until precipitation occurred. An equal volume of $(NH_4)_2 SO_4$ was necessary for this to occur. The resultant mixture was left at room temperature for 10 minutes and centrifuged at 15000 rpm for 15 minutes. The supernatant was discarded and the precipitate resuspended in 0,15 M saline. The ammonium sulphate precipitation step was repeated. The resuspended IgG was dialysed overnight in a large volume of saline containing sodium azide to remove the residual $(NH_4)_2 SO_4$ and then adjusted to the original volume. (Hardie and van Regenmortel, 1977).

c) Further purification of Gamma Globulin.

The host absorbed IgG was further purified by ion exchange chromatography by filtration through a column (15cm x 1cm) containing diethylaminoethyl (DEAE) cellulose (Whatman DE 52 anion exchanger). The DEAE cellulose column was pre-equilibrated with half-strength PBS (pH 7,4) (IX.A.1(b)). The gamma globulin was washed through the column with the same buffer. One millilitre fractions were collected and the eluent monitored by UV absorption at 280nm. The first protein fractions were collected and pooled, and their UV absorbance at

280nm measured. The gamma globulin fraction was adjusted to a concentration of 1 mg/ml with half-strength PBS (pH 7,4) (IX.A.1(b)). The extinction coefficient of gamma globulin was taken as $E^{0,1\%}_{280nm} = 1,4$ (Clark and Adams, 1977).

d) Conjugation with Alkaline Phosphate.

Two millilitres of purified immunoglobulin (1 mg/ml) was mixed with 2 mg/ml alkaline phosphatase (Seravac, salt-free, freeze dried from beef mucosa). This was dialysed overnight at 4°C against two litres of half-strength PBS. The contents of the dialysis bag were then transfered to a small vial and 2,5% (v/v) gluteraldehyde (Merck, 25%) added. This was left at room temperature (22°C) for 4 hours and then dialysed overnight against two litres of half-strength PBS to remove the gluteraldehyde. BSA was added to a final concentration of 1% to stabilize the conjugate which was stored in a sealed bottle at 4°C.

The optimal concentration of the immunoglobulin and conjugate were determined using the indirect ELISA technique (IX.D.5(a)(ii)). By varying the antibody and conjugate dilutions and virus concentration, the best conditions for subsequent assays could be calculated. The optimum combination of each would ideally give a broad range of absorbance (at 405nm) readings (IX.B).

5. Enzyme-linked immunosorbent assay (ELISA).

a) Assay procedure

(i) DAS-ELISA

The wells of Nunclon microtitre plates (Microwell Plate 96F, Weil Organisation) were coated with 200 ul of purified gamma globulin at pre-determined optimal concentrations for antigen detection. Dilutions of IgG were made in coating buffer (IX.A.3(a)). The microtitre tray was incubated in a closed moist plastic container in a Memmert incubator set at 37°C for 1,5 - 2 hours. The wells were emptied and washed by flooding the tray with rinsing buffer (IX.A.3(b)). The washing procedure consisted of three washes of 10 minutes each in rinsing buffer, followed by incubation of the tray, flooded with post-coating buffer containing BSA (IX.A.3(c)) for 15 minutes at room temperature. This minimized non-specfic adsorption of antigens to the wells since all unbound sites were occupied by BSA. The wells were drained and the trays dried by strong tapping on a paper towel. The sample was diluted in post-coating buffer (IX.A.3(c)) and 200 ul of each dilution added to the appropriate well of the ELISA tray. The plate was then incubated for 1,5 - 2 hours at 37°C as

described previously. After this time the tray was washed, drained and 200 ul of the alkaline phosphatase-conjugated globulin (IX.D.4(d)) at the appropriate dilution in conjugate buffer (IX.A.3(c)) added to each well. The tray was incubated at 37°C for 1 hour or at 4°C overnight. The wells were washed again and 300 ul of substrate (IX.A.3(d/e)) added to each well. The tray was left at room temperature for the enzyme reaction to take place. The colour reaction which occurred was quantitated by reading absorbance at 405nm using a Titertek Multiskan automatic read-out spectrophotometer (Type 310C, Flow Laboratories).

(ii) Indirect ELISA

The "indirect" ELISA is a variant of the DAS-ELISA technique. The wells of the microtitre tray were coated with antigen (purified virus) diluted in PBS (pH 7,4) (IX.A.1(b)), incubated and washed as described for DAS-ELISA (IX.D.5(a)). After incubation with post-coating buffer (IX.A.3(c)), the wells were coated with antibody also diluted in post-coating buffer, incubated and washed. Goat anti-rabbit alkaline phosphatase conjugate at a dilution of $^{1}/_{750}$ in post-coating buffer was used. After incubation and washing, 300ul of substrate were added to each well and the colour reaction monitored as in the DAS-ELISA (IX.D.5(a)).

b) Controls

In general healthy plant sap or healthy, uninfected, homogenized protoplasts constituted negative antigen controls. Reactions caused by non-specific colour development were monitored by including a set of wells containing:

(i) exclusion of one component from the procedure i.e. antibody, antigen of conjugated components.

(ii) substrate only.

Negative controls generally had an OD 405nm reading of not higher than 0,05 - 0,1 OD unit.

6. Polyacrylamide gel electrophoresis (PAGE).

PAGE electrophoresis was performed according to the method of Laemmli (1970). The buffers and solutions used for preparing the acrylamide gel and samples appear in the Material section (IX.A).

a) Preparation of Resolving Gel.

A 12,5% polyacrylamide gel was prepared by mixing the following (quantities suffient for 2 gels):

Solution	Volume(ml)
acrylamide : bis-acrylamide (IX.A.4(d))	33
IM Tris/HCl pH 8,8 (IX.A.4(a))	30
10% SDS (IX.A.4(f))	0,8
1,5% ammonium persulphate (IX.A.4(g))	4,0
water	12,2
Temed (IX.A.4(h))	0,02

The vertical slab gel apparatus was prepared using glass plates (18cm x 16cm) (cleaned with ethanol) and plastic spacers (1,5mm). Once the resolving gel had been mixed, it was immediately dispensed between the glass plates and overlayed with water or isopropanol so that a perfectly straight meniscus was formed. The gel thus prepared was approximately 1,5mm thick and 100 - 110mm in length. It was allowed to set at room temperature, the water/isopropanol poured off and replaced by the stacking gel.

b) Preparation of stacking gel.

A 4,5% stacking gel was prepared as follows (quantities sufficient for 2 gels):

Solution	Volume(ml)
acrylamide : bis-acrylamide (IX.A.4(d))	2,25
IM Tris pH 6,8 (IX.A.4(b))	1,9
water	9,0
80% glycerol	1,0
10% SDS (IX.A.4(f))	0,5
1,5% ammonium persulphate (IX.A.48(g))	0,7
Temed	0,02

10ml of the above stacking gel mixture was dispensed on top of the resolving gel and a 10 well comb inserted. Once the gel was set, the comb was carefully removed. The sample wells were then covered in running buffer (IX.A.4(c)).

c) Sample preparation.

Samples to be electrophoresed were mixed with an equal volume of dissociation mixture (IX.A.4(e)). These were then heated for 10 - 15 minutes at 100°C.

d) Sample application and electrophoresis.

Samples were carefully loaded into the sample wells of the stacking gel using a 25 or 100ul Hamilton syringe. The gel was then assembled in a Hoefer SE600 vertical slab electrophoresis unit. Electrophoresis was performed by connecting the upper buffer chamber to the cathode and the lower buffer chamber to the anode. The whole operation was carried out at 4°C. A constant current of 8mA per gel was applied for 16 hours after which time the glycinate ion front containing the bromo-phenol blue had migrated to within 1cm of the bottom of the gel.

e) Staining/Destaining.

Gels were stained by immersion overnight in stain containing 0,02% (w/v) Coomassie brilliant blue (BDH Chemicals, England.) (IX.A.4(i)). Destaining of the gels was carried out by soaking the gel in several changes of destain (IX.A.4(j)). The destained gels were dried on to filter paper (Whatman No. 3) using a Hoefer gel drying apparatus (Hoefer Scientific Instruments, San Francisco).

7. Immuno-electroblotting

The procedure for electro-blotting was essentially that of Towbin *et al* (1979) and adapted for plant virus proteins by Rybicki and von Wechmar (1982).

a) Electrophoretic transfer.

Samples to be electroblotted were first electrophoresed on vertical slab acrylamide gels as described in Section IX.D.6.

The stacking gel was cut away and the resolving gel was laid on nitrocellulose sheets(Schleider and Schuell, BA 85, 0,45um pore) and sandwiched between wetted filter paper/sheets (Whatman No. 3). The gel sandwiches were placed in the assembly unit of a Hoefer Scientific Instrument (San Francisco) Model TE 50 apparatus. Two gels could be accommodated in one assembly unit. This was completely immersed in a 5 litre capacity tank containing transfer buffer (IX.A.5(a)). The nitrocellulose sheets were assembled so that they were closest to the cathode side of the apparatus. A current of 0,5 - 1 volt was applied for 1,5 - 2 hours. There usually was an increase in the tank transfer buffer temperature. This did not seem to hinder transfer of bands from the gels to the nitrocellulose paper, but the problem could be alleviated by electroblotting at $4^{o}C$, by setting up the system in a Coldroom.

b) Enzyme-assisted indirect immunoassay.

Following the electroblotting step(IX.D.7(a) above) the nitrocellulose paper was removed and soaked overnight at 22°C in soaking buffer (IX.A.5(b)) to saturate free protein binding sites with BSA. Either rabbit antisera or host-absorbed IgG was diluted $^{1}/_{25}$ - $^{1}/_{100}$ in the same buffer. The nitrocellulose sheets were immersed in the diluted antisera/IgG and incubated on a shaker at 22°C for $1^{1}/_{2}$ - 2 hours. The blots were then washed for 10 minutes in a shaker in at least 4 changes of washing solution (IX.A.5(c)).

Goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad Laboratories, EIA purity grade) was diluted $^{1}/_{1500}$ in soaking buffer (IX.A.5(b)) and incubated with the blots for 1 - 2 hours at 22°C on a shaker. After further washing in washing solution as described before, the enzyme substrate solution (IX.A.5(d)) was added. The colour reaction was stopped by washing the blots in deionized water. Blots were dried between sheets of filter paper (Whatman No. 3) before photographing.

8. Electron microscpoy

a) Negative staining

A drop of virus sample was placed on a carbon coated grid resting on parafilm. The grid was covered with a Petri dish and left for 15 minutes at room temperature. The grid was washed with a series of drops of distilled water from a Pasteur pipette. Grids were stained for 1 minute with 2% Uranyl acetate pH 5,0 (IX.A.10(a)). The grid was drained by touching the edge with a piece of filter paper and allowed to dry. The grid was viewed in a Zeiss 109 transmission electron microscope at 80 KV.

b) Immunosorbent Electron Microscopy (ISEM)

The technique is also known as serologiocally specific electron microscopy or the Derrick method and was first described by Derrick (1973) for use with plant viruses. The technique has been used extensively in the detection, quantitation and characterization of plant viruses (Derrick and Brlarsky, 1976).

The technique used is the short incubation method of Milne (1980). Five microlitres of antiserum, diluted 1/1 000 in PBS (IX.A.1.(b)), were placed on a piece of Parafilm in a humid Petri dish. The grids were incubated face down on those drops for five minutes. The grid was then rinsed with 20 drops of 0,1 M sodium

phosphate buffer, pH 7,0 (IX.A.1.a) and drained by touching the edge with filter paper. The grid was then incubated face down for 15 minutes on a drop of the virus suspension. The grid face was washed with thirty drops of distilled water from a Pasteur pipette and stained for 1 minute with 2% uranyl acetate (IX.A.10 (a)). It was drained as before, allowed to dry and viewed.

c) Decorating

Carbon-coated grids were coated with a 1:1 mixture of anti-MDMV and anti-CMV sera which had been diluted 1/500 as described for ISEM (IX.D.8(b)). They were incubated for 1,5 hours at $4^{o}C$ on a drop of antigen, in this case the sap of maize, doubly infected with MDMV and CMV. After washing in 10 drops of PBS (IX.A.1(b)) the grids were incubated for 1 hour at $22^{o}C$ with anti-MDMV serum at a 1/250 dilution, washed, negatively stained with 2% uranyl acetate and viewed.

9. Isolation of RNA

For the extraction of RNA from plant viruses all buffers, pipette tips and Eppendorf tubes must be autopclaved at 15 lbs/in^2 for 15 minutes before use.

a) Extraction

MDMV-B-ST was used at a concentration of 4 mg/ml and CMV-K at 2 mg/ml. One hundred microlitres of the freshly purified virus was disrupted in 250 ul of disruption mix (IX.A.9(a)) at 65°C for 2,5 minutes in a sterile Eppendorf tube. Five hundred microlitres of phenol was added and the tube shaken quickly to mix the aqueous and phenolic phases. Centrifugation for 5 minutes in an Eppendorf Centrifuge (Model 5413) was carried out. The aqueous phase was removed carefully and transferred to a new sterile Eppendorf microtube. The phenol extraction was repeated until the aqueous phase was devoid of protein precipitate. Two and a half volumes of ethanol were added to the aqueous phase in a new Eppendorf and the mixture adjusted to a 4% salt concenration by the addition of 60 ul 3 M sodium acetate (IX.A.9 (d)). The mixture was left at -20°C for at least 2 hours to precipitate the isolated RNA. Centrifugation at 4°C in a Eppendorf centrifuge 5413 for 10 minutes was carried out and the ethanol removed firstly by pipetting and then by evaporation under vacuum in a Speed Vac concentrator (Savart Instruments,

New York) for 15 minutes. The resulting RNA pellet was resuspended in 100 ul of resuspension buffer (IX.A.9 (c)) and heated for 1,5 minutes at 60°C. It was cooled on ice before loading onto a 1,5% agarose gel.

b) Preparation of agarose gels

The running plate of a Hoefer HE33 'Minnie' Horizontal Submarine Unit (Hoefer Scientific Instruments, San Francisco) was cleaned using commercial methanol and positioned in a casting tray on a level surface so that the handles of the running plate rested on the rim of the casting tray. Sterile 1,5% agarose (IX.A.9(f)) in TBE buffer (IX.A.9(b)) was heated on a heating mantle until melted. When the agarose was 50°C, approximately 25 ml were poured into the casting tray on top of the running plate. An eight-well comb was inserted so that the support bar of the comb rested on the edges of the casting tray. The gel was approximately 3 mm thick. It was left to solidify for approximately 1/2 hour. The running plate and gel were removed from the casting tray and placed on the centre platform of the electrophoresis unit. The comb was carefully removed and the unit filled with sterile running buffer (IX.A.9 (b)) so that there was about a 1 mm layer of buffer over the gel.

(c) Sample application and electrophoresis

The gel was "pre-run" for 15 minutes at 50 volts before loading samples. Extracted RNA samples were loaded into the sample wells using a micropipette and sterile pipette tips. A molecular weight marker was introduced (Electran, M_r range 4S-28S; BDH Chemicals, England). The lid was placed on the assembled unit so that the cathode was positioned at the end closest to the sample wells. The apparatus was connected to a power supply and electrophoresis was carried out at 50 volts for 1,5-2,5 hours. The gel was stained for 15 minutes with ethidium bromide and viewed on a transilluminator (UV products Inc., USA). Gloves were used for handling the gel once ethidium bromide had been added and goggles were worn when viewing the gel. If the gel was overstained, it could be destained by immersing in 0,01 M $MgCl_2$ (IX.A.9 (g)) for 5-10 minutes.

10. Radiolabelling of cucumber mosaic virus

"^{35}SLR General purpose" ^{35}S labelling reagent (t-butoxycarbonyl-L-[^{35}S] methionine N-hydroxysuccinimidyl ester) was used for radiolabelling viruses. The method is summarized below.

Ten uCi of ^{35}SLR were added to an Eppendorf microtube. A stream of nitrogen gas was gently directed on to the surface of the radiolabel to evaporate the solvent in which the label was stored. The reaction tube was cooled on ice and 1 mg/ml CMV-K or BMV-ST in 0,1 M borate buffer, pH 8,0 (IX.A.2(b)) was added. After 30 minutes the reaction was stopped using 100 ul of 0,2 M glycine in 0,1 M borate buffer pH 8,0. The labelled virus preparations were subjected to PAGE gel electrophoresis (IX.D.6) and autoradiography (IX.D.11).

11. Autoradiography

Samples for autoradiography were subjected to PAGE gel electrophoresis (IX.D.6). The acrylamide gels were stained for three hours in Coomassie stain (IX.A.4 (i)) and destained for 4 hours at 37°C in destain (IX.A.4 (j)). The gels were dried for 1,5 hours on a Hoefer gel apparatus (Hoefer Scientific Intruments, San Francisco) which was reserved for radioactive gels. The dried gel was taped firmly into a sealed cassette (Okamoto, Japan) and an X ray film (X-Omat AR film, Kodak) taped firmly over the top of it. The cassette was stored at -70°C for 5 days. The exposed X-ray film was developed in the dark using only a red light, by immersng it in Kodak X-ray developer for 5 minutes, transferring it to water for two minutes and then to Kodak X-ray fixer for a further 5 minutes. The X-ray film was washed under running water for approximately 30 minutes before hanging up to dry.

12. Evans blue staining

Evans blue staining was used to assess the viability of protoplasts. Lethally damaged cells can be visualised by their failure to exclude the dyes (Gaff and Okongo'o-Ogala, 1971). One drop of 0,025% (w/v) Evans' blue (Gurr, BDH Chemicals) in 0,7 M mannitol was added to one drop of the protoplasts on a haemocytometer. After 5-10 minutes the surviving protoplasts were counted.

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